Respiratory Syncytial Virus Bronchiolitis and Disease Severity

From molecular profiles to clinical biomarkers

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Chapter 1

General introduction

RSV LOWER RESPIRATORY TRACT INFECTIONS

Lower respiratory tract infections (LRTIs) are among the most frequent indications for hospitalization and form worldwide the leading cause of death in children under 5 years of age.[1] Respiratory syncytial virus (RSV) is the most important cause of respiratory tract infections in infants and young children worldwide and a major reason for hospitalization.[2] Clinical manifestations of RSV infections range from mild upper respiratory tract symptoms such as cough, rhinorrhea and conjunctivitis to severe LRTI and in its most severe form life-threatening respiratory insufficiency requiring mechanical ventilation. Treatment for LRTI by RSV is largely supportive and a vaccine is not yet available, although several phase 2 and 3 studies are ongoing.[3, 4]

The annual global incidence of LRTIs caused by RSV among children younger than 5 years, has been estimated at 34 million per year leading to at least 3.4 million hospital admissions and up to 199.000 deaths, the majority in developing countries.[2] Most children have been exposed to RSV by the age of 2 years. Approximately 1 to 2% of all children infected by RSV (10% of those with lower respiratory tract involvement) are hospitalized and about 6-11% of these children require mechanical ventilation for a severe RSV LRTI.[5, 6] Meijboom et al. recently estimated the burden of RSV disease in children below the age of 12 months in the Netherlands, and reported almost 29.000 RSV related visits to the general practitioner, 1623 hospitalizations, and 5 deaths.[7] Hence, RSV infection is the most frequent cause of non-elective pediatric intensive care unit (PICU) admission for mechanical ventilatory support in infants during the winter season resulting in annual capacity problems at the PICUs in the winter months.

DISEASE SEVERITY

Risk factors for severe RSV infection – including prematurity, chronic lung disease, congenital heart disease and immune deficiency – have been well described. However, more than half of the RSV-infected children requiring intensive care admission are otherwise healthy.[5, 8] It is unclear why some children develop a more severe course of disease than others.

Disease severity is the result of a dynamic interplay between direct viral damage and the immune response of the host. Upon infection, virus specific structural components are recognized by pattern recognition receptors of the host, triggering mechanisms to suppress viral replication and elimination of viral infected cells. At the same time, however, this immune response also results

in inflammation of the respiratory tract and determines the extent of disease severity.[9]

It is not completely clear whether severe manifestations of lower respiratory tract disease are caused by directly induced viral damage or by the immune response of the host. In addition, there is little agreement whether disease severity is associated with a defective/immature or an excessive immune response. While some studies show the presence of a strong inflammatory response characterized by abundant inflammatory cytokines and activated granulocytes in the airways of infants and children with severe RSV disease [10-15], others report decreased concentrations of inflammatory cytokines and other markers of cell injury in the more severe phenotypes of RSV disease.[16-18]

Assessment of Disease Severity

To date, physicians have few tools to predict disease severity and outcome in the individual patient. The decision to admit a young infant with bronchiolitis to the hospital is based on clinical parameters combined with an assessment of risk factors for severe disease. However, 35% of the children hospitalized with bronchiolitis do not require supportive interventions during hospitalization [19] and 4.6-6.8% of the patients initially discharged from the outpatient clinic, require hospitalization later on during the course of infection.[20, 21]

To improve patient management, laboratory tests identifying those patients who are at risk for a severe course of disease and who require supportive interventions and/or intensive care admission are needed. Alternatively, accurate selection of patients with milder manifestations of disease could prevent unnecessary hospitalizations and reduce health care costs. At this moment no clinical tools are available that allow differentiation between these patient groups. In our opinion, there is a need to develop biomarkers that may help clinicians to select those patients who can be safely discharged home or who require close monitoring and/or supportive interventions in their course of disease.

AIM OF THE THESIS

The aim of this thesis is to identify diagnostic biomarkers that have the potential to differentiate between mild and severe cases of RSV-caused bronchiolitis. Viral factors and host immune response patterns are evaluated in young children with viral bronchiolitis to select markers that may differentiate between mild and severe disease and may be used as future biomarkers for disease severity.

OUTLINE OF THE THESIS

Chapter 2 provides a general introduction to the field of biomarkers in pediatric infectious diseases. Recent advances in the application of genomics and proteomics to develop biomarkers for infectious diseases are described and current clinical applications and future perspectives are discussed.
Viral factors and host immune responses contribute to disease severity and may be used as biomarkers. In **chapter 3**, we evaluate the role of viral factors such as infection by multiple viral pathogens or viral load in disease severity in young children with bronchiolitis.
In the subsequent chapters specific host immune factors are analyzed for their ability to discriminate between mild and severe disease in young children with viral bronchiolitis. In **chapter 4**, we explore the role of the matrix metalloproteinases MMP-8 and MMP-9 as biological markers to assess disease severity in viral lower respiratory tract infections in children. We additionally investigated in **chapter 5** if disease severity in RSV-infected infants can be distinguished by the use of combinations of several immunological markers.
In the last part of this thesis, proteomic and transcriptomic technologies are used to identify proteins and genes which may be potential targets for the development of future therapy or prevention of severe viral respiratory diseases. In **chapter 6** we aimed to identify genes that can be used as novel biomarkers for disease severity using transcriptomic analyses of human leukocyte subsets obtained from children with LRTIs. In **chapter 7**, an in vitro study was performed to describe the effect of respiratory virus infection on protein expression levels in human airway epithelial cells by 2-D DIGE analyses. In **chapter 8**, an experimental murine infection model was used to study the molecular mechanism underlying vaccine induced RSV-specific enhanced disease by applying mass spectrometry assisted protein profiling.
Finally, in **chapter 9** we discuss the results of our studies, a short update on the use of proteomics and transcriptomics in RSV research is provided and perspectives for further research are presented.

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Chapter 2

Host biomarkers and pediatric infectious diseases; from molecular profiles to clinical application

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INTRODUCTION

Infectious diseases are an important cause of death among children under the age of 5.[1] Most of these deaths are caused by preventable or curable infections. Limited access to medical care, antibiotics, and vaccinations remains a major problem in developing countries. But infectious diseases also continue to be an important public health issue in developed countries. With the help of modern technologies, some infections have been effectively controlled; however, new diseases such as SARS and West Nile virus infections are constantly emerging. In addition, other diseases such as malaria, tuberculosis, and bacterial pneumonia are increasingly resistant to antimicrobial treatment.

The physician who manages pediatric patients with infectious diseases is confronted with several related challenges. First, one should establish a specific diagnosis, preferably early in the course of disease. Despite improvements in culture and non-culture diagnostics, in many cases, the causative microorganism remains unknown. Consequent delays in initiation of appropriate treatment can contribute to the emergence of antibiotic resistance.

A second challenge is to identify those patients most likely to develop severe disease. To date, physicians have little information on prognosis and disease outcome in the individual patient. It would be extremely useful to be able to identify patients at risk for more severe disease (e.g., secondary bacterial infection during viral respiratory tract infection), as such prediction could inform management decisions. The third associated challenge is to select the most appropriate treatment strategy for an individual patient. While some patients require intensive support, others will recover without additional medication or supportive care. To date, few tools are available to monitor the course of disease after initiation of medical treatment.

Biomarkers have been used for years to help clinical decision-making. C-reactive protein (CRP) is probably the best known marker used to monitor infection. Although useful, it does not reliably distinguish viral from bacterial infections. More recently-developed markers such as procalcitonin seem promising, with detectable rises early in the course of infection and high negative predictive value as seen in children with fever of unknown origin.[2-4] However, this marker has also insufficient power to discriminate between viral and bacterial infections. Additionally, these conventional biomarkers for infectious diseases do not provide microorganism-specific prognostic information.

The completion of the Human Genome Project and the introduction of powerful DNA microarray chips and proteomic technologies in the mid-1990s have created

the opportunity to identify genes and proteins that may serve as biomarkers in infectious diseases. The identification of biomarkers may enable the development of exciting potential clinical applications in which genes and proteins that are differentially expressed in healthy and infected individuals can be investigated.[5] These approaches may provide detailed insight into the pathogenesis of disease, host pathogen interactions, and disease-specific expression patterns. In addition, diagnostic and prognostic biomarkers and markers that monitor disease or response to therapy may be developed using these technologies.
This chapter describes recent advances in genomics and proteomics in the field of biomarkers for infectious diseases and summarizes current clinical applications and future perspectives.

MOLECULAR PROFILING: CURRENT TECHNIQUES

Genomics

Single nucleotide polymorphisms (SNPs), single base pair changes at specific spots in the genome, are the most common type of genetic variation. The human genome carries over 10 million nucleotides that vary in at least 1% of the population. Currently, approximately 6 million nucleotides have been validated and this number is still growing.[6, 7] Although SNPs are the changes most frequently explored using high throughput technologies, other genetic variations are also common in the human genome and may influence the individual's susceptibility to disease. These include variations in gene copy number, repeating sequence motifs, insertions, and deletions.[8]
The completion of the human genome map in combination with the development of microarray-based comparative genomic hybridization and genome-wide SNP platforms have permitted the screening of the entire genome to identify genetic loci linked to certain diseases, susceptibility to disease or response to therapy.[9] These genome wide association studies allow identification of genetic risk factors for a wide variety of common and more complex diseases by measuring hundreds of thousands of genetic variants simultaneously. Using SNP platforms, MalariaGen, a genomic epidemiological network, and the Wellcome Trust Case-Control Consortium (WTCCC) have performed a large scale study on disease susceptibility.[10, 11] Both consortia have analyzed up to 500,000 SNPs in thousands of African individuals diagnosed with malaria or tuberculosis between 2006 and 2008. In addition, the WTCCC will include approximately 2,000 cases and

3,000 controls for 8 other diseases, which makes it one of the biggest projects aimed at the identification of genetic variations that may predispose a patient to disease.[12] These genome-wide analyses may contribute to the identification of individuals at risk and produce more effective prevention strategies and individual treatment strategies.

Transcriptomics

Where genomics provides information on genetic susceptibility to certain infectious diseases, transcriptomics provides information on the activity of genes at a certain moment under certain conditions. It is the study of the complete set of RNA transcripts produced by the genome. During all biological processes, part of the genome is specifically transcribed into messenger RNA (mRNA, transcriptome) and translated to proteins (proteome). The transcriptome can be analyzed using gene expression microarrays. These chips contain either the whole genome or a subset of specific genes. mRNA is extracted from experimental samples, reverse transcribed and labeled with fluorescent dyes. The extracted labeled cDNA is then hybridized with the microarray and the fluorescence of the array is determined using an array scanner. Following image analysis, the data are subjected to bioinformatics processes to identify statistically significant changes in gene expression between different samples. The technique can be used to characterize gene expression in both pathogen and host, providing detailed insight into host-pathogen interactions during infection.[13] To study them on a molecular level, Kawada et al. generated gene-expression profiles in peripheral blood mononuclear cells (PBMCs) isolated from children with influenza virus infection.[14] Many genes associated with the immune response such as interferon regulated genes appeared to be strongly upregulated during influenza infection. In addition, they compared gene expression profiles of influenza-infected children with and without convulsions. They found that transcription levels of pro-inflammatory cytokine genes in patients with a febrile convulsion were not significantly different from those in patients without febrile convulsion. This kind of approach may help to clarify the pathogenesis of influenza and its neurological complications.[14]

Proteomics

While gene expression profiles may not completely correlate with intracellular protein content, proteomics can provide insight into the structure and dynamics

of the end product, proteins. Proteomics is the study of the proteome, the complete set of proteins, their modifications, interactions, and localization. Proteomic technologies enable detailed analyses of protein expression and evaluation of post-translational modification and protein stability and turnover that cannot be assessed by genomic and transcriptomic profiling alone. For many years, two-dimensional gel electrophoresis has been the standard technology to isolate specific proteins and allow protein identification by subsequent mass spectrometry. During the past decade, mass spectrometry has improved and now enables the analysis of protein expression, structure, and function without the need for labor intensive and time consuming electrophoresis.[15, 16] In addition, several mass spectrometry-based approaches have been developed that allow the relative or absolute quantification of proteins.

In an attempt to identify biomarkers and to develop screening tools with high sensitivity and specificity, proteomics technology has been applied to analyze biofluids such as serum, saliva, or urine. A commonly used technique is a surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF), which is a proteomics technique that allows the identification of large numbers of proteins in a short period of time. This technique provides a specific mass spectral profile from each analyzed protein sample. By comparing profiles from affected individuals to those derived from healthy controls, a specific protein signature is obtained.[17, 18] Agranoff and colleagues have used it to characterise distinct profiles for several microbial infections and to investigate serum responsiveness to *M. tuberculosis* identifying serum biomarkers from patients with advanced tuberculosis.[19] SELDI-TOF has the potential to identify tuberculosis at an early stage, assisting early diagnosis and therapy, which is important for favorable outcome.[19]

CLINICAL APPLICATIONS IN PEDIATRIC INFECTIOUS DISEASES

The recent advances in proteomic and genomic technologies have allowed the identification of genes and proteins that may serve as biomarkers for the diagnosis and monitoring of infectious diseases. Application of knowledge from these technologies into clinical practice is still at an early stage. In the following sections, we will first discuss current literature on the contribution of the aforementioned technologies for the determination of disease pathogenesis, for the susceptibility to infection, and for diagnostics. In the next section, we will discuss clinical applications of proteomic and transcriptomic technologies, and,

in the last section, we will focus on their future perspectives.

What Has Been Studied up to now?

With increasing use of genomic and proteomic technologies, more insight has been obtained into host-pathogen interactions and pathogenesis of infectious diseases. The response of the host to pathogens is reflected in changes in gene expression and can be measured by microarray based gene expression technologies. Likewise, transcriptional profiling studies have proven to be a powerful approach for analyzing and understanding host–pathogen interactions. Based upon the host response to various pathogens, Jenner et al. have identified common and more specific gene expression patterns.[20] They collected and systematically compared transcriptional profiling datasets from 32 published microarray-based in vitro studies which collectively examined 77 different host-pathogen interactions. In response to this wide variety of pathogens, they identified a cluster of 511 genes that share a common response upon infection with a pathogen. According to the localization of the cell in which they function, these genes have been clustered into different functional groups in order to provide an overview of cellular physiology involved in the common host response. Moreover, analyzing different transcriptional profiling studies also revealed pathogen specific gene expression patterns. Several host genes of the aforementioned common host response were found to be downregulated in the presence of pathogens or specific pathogen proteins. These transcriptional profiling studies has provided insight into how microorganisms alter host gene expression patterns to subvert the immune responses. For example, transcriptional profiling has identified that viruses such as herpes simplex virus (HSV)-1, human cytomegalovirus, and human papillomavirus-31 are partially or completely able to inhibit the induction of Interferon stimulated genes, which have a central role in the defense against viruses.[20]

Streptococcus pneumoniae and influenza virus are the most common causes of pneumonia. Consequently, they contribute to substantial morbidity and mortality worldwide. It has been known for years that influenza infection predisposes to secondary bacterial infection, mainly caused by *S. pneumoniae* and *S. aureus*. The catastrophic influenza A pandemic in 1918 in which approximately 40–50 million persons were killed, may have involved synergy between influenza and pneumococcal infections. Gene expression profiling is a powerful tool to explore the molecular mechanism of synergy between pathogens. By host gene expression analyses of the lungs in mice, Rosseau et al. differentiated pneumococcal

infections from influenza. Rosseau et al. have also identified common gene expression patterns in infectious disease as well as unique pathogen-specific gene expression signatures that may help clarify the mechanisms behind the synergy between influenza virus and *S. pneumoniae*.[21] In response to influenza infection, Tong et al. performed gene expression analyses of middle ear epithelial cells.[22] They suggest that increased expression of inflammatory mediator genes such as Interferon gamma-induced protein (IP-)10 and C-X-C motif chemokine (CXCL-)11 could lead to a shift in *S. pneumoniae* adherence by activation of host epithelial and endothelial cells, providing a favorable environment in the middle ear cavity for a secondary bacterial infection with *S. pneumoniae*.

The response upon exposure to pathogens varies widely between individuals. Some people are more susceptible to a certain infection than others. This differential susceptibility is partly caused by genetic variations between individuals that may predispose either to development of disease or to a more severe course. Although the large variation in clinical responses among individuals, also depends upon environmental and microbial factors. The classic example of host genetic susceptibility is the resistance of heterozygous hemoglobin S individuals to malaria infection (caused by *Plasmodium falciparum*).[23] Other approaches used to elucidate genetic and environmental effects on infections include studies in identical and non-identical twins and comparisons of risk in adopted children and their biological and adoptive parents.[24-26] One such study suggested that adopted children with a biological parent who died early of an infectious disease had a higher risk of mortality from similar infections while the death of an adoptive parent due to infection had no influence on the risk of disease in the children.[27]

Recent advances in microarray technologies have enabled genome wide searches for genes influencing susceptibility to infectious diseases. Analysis of genetic susceptibility aims to link the genetic code (genotype) to the risk of a certain disease state (phenotype). Given the large number of human genes, many with unknown function, genome wide studies have the advantage that previously unconsidered genes can be identified and provide more sensitivity for the detection of subtle genetic effects and gene recruitment in affected individuals. However, many reported genetic associations have not been replicated in subsequent studies, and, for secure results, large numbers of affected and unaffected individuals are required. Furthermore, because of the complexity of data analysis, microarray technologies are time and labor intensive.[28-30] Nevertheless, several large scale population based studies have been performed and support the role of genome wide searches in the identification of genes influencing disease susceptibility.[11,

[31-33] For example, a genetic association study performed by O'Brien et al. has led to the identification of various genetic factors that affect HIV-1/AIDS.[31] Genetic association analysis of several large cohorts of HIV infected individuals resulted in the identification of 14 AIDS restriction genes, which are human genes with polymorphic variants that influence the outcome of HIV-1 exposure or infection. This study illustrates the discovery of previously unknown genes involved in susceptibility to infection using SNP haplotype-based association studies in clinically well-described epidemiological cohorts.

Another potential application of microarray technologies is diagnosis of infection both by direct and indirect identification of pathogens. For example, microarrays composed of DNA sequences of various pathogens allow the identification of many organisms in a single test. Wang et al. developed an array composed of all fully sequenced reference viral genomes that allows the detection of approximately 1000 viruses.[34] Moreover, by sequencing hybridized material of unknown pathogens, this array permits identification of new viruses, and, in 2003, it proved successful in the global effort to identify the novel corona virus associated with severe acute respiratory syndrome (SARS).[34, 35]

In contrast to direct identification, infections can also be characterised indirectly through specific host responses. An advantage of such pathogen-specific molecular signatures in the host is that they may be present at various stages of infection, even when the pathogen is not detectable using standard or direct diagnostic tests. Ramilo et al. used gene expression analysis to diagnose different pathogen fingerprints in pediatric patients with respiratory infections caused by influenza A virus and Gram-negative (*E.coli*) or Gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*) bacteria.[36] Classifier genes, which discriminate influenza A from bacterial infections (*S. pneumoniae* and *S. aureus*) and *E. coli* from *S. aureus* infections, were identified and validated. Another example of "-omic" technology use in diagnosing infection is the development of a protein based signature for diagnosing trypanosomiasis or sleeping sickness, which affects half a million people yearly in sub-Saharan Africa. Trypanosomiasis, if left untreated, is a debilitating disease with a lethal outcome; it was successfully controlled in the past, but, since the 1970s, has re-emerged as an epidemic of immense proportions causing huge, yet widely underestimated morbidity and mortality of up to 50,000 cases every year.[37] Establishing the diagnosis remains complicated, as current diagnostic tests lack the sensitivity to detect low parasite loads in peripheral blood. Papadopoulos et al. analyzed serum samples from patients and controls using SELDI-TOF mass spectrometry and identified distinct serum proteomic signatures in both groups.[38] After depleting

serum samples of antibody components, the authors identified two prominent protein peaks at 23/24 and 47 kDa in patients. These proteomic signatures may provide the basis for new diagnostic tests and alternative methods to monitor the host response to treatment. Moreover, additional characterization of these differentially expressed proteins may allow the development of simpler, cheaper antibody based tests.[19,38]

Current and Potential Clinical Applications of "-omic" Technologies

More than 50% of all children admitted to the hospital have fever or other nonspecific symptoms related to infection.[39] Although not necessarily suffering from bacterial infection, a significant proportion of these children will receive antibiotics. Although clinical history, physical examination and conventional diagnostic investigations (e.g., x-rays, blood tests) may point to an extent towards cause, pathogen identification remains difficult or even impossible. During episodes of acute fever, clinicians prefer to rely on cultures taken from the site of infection. However, such cultures often cannot be obtained at the right time or from the relevant site (e.g., middle ear or lungs) and results are not available for at least 24 h after sampling so that pathogens often remain undetected. Furthermore, contamination and colonization, particularly in upper airway samples, can obscure results. Gene expression profiles may identify bacterial pathogens and discriminate between bacterial infections, infections caused by other pathogens, and non-infectious causes of fever (like auto-inflammatory diseases). Using microarrays, organisms can be identified either directly or indirectly through their effects on host gene expression.

Tang et al. showed that gene expression profiling of neutrophils can distinguish sepsis from non-infectious inflammation (e.g. Systematic Inflammatory Response Syndrome, SIRS) in intensive care patients.[40] They performed microarrays on a cohort of septic (N=71) and non-septic (N=23) patients and identified 50 classifier genes differentially expressed between the two groups, which are involved in inflammatory responses, immune regulation, and mitochondrial function. Broadly genes involved in the upregulation of immune responses were expressed less in patients with sepsis than in control patients, whereas genes involved in down regulation of immune responses were expressed more, suggesting that sepsis may have an inhibitory effect on immune regulation. Pathway analyses support the finding that immune regulation is inhibited during sepsis by showing that genes involved in the NF-B pathway were expressed less in patients with sepsis, whereas the inhibitory gene NFKBIA was expressed more. A prediction model

for disease severity was developed from these data and validated in a second more heterogeneous patient group.[40] A major advantage of gene expression profiling is that it may enable the development of less expensive diagnostic tools such as quantitative real-time polymerase chain reaction (RT-PCR) detection and quantification of specific DNA sequences in septic patients instead of entire gene expression profiles.

Children with auto-inflammatory diseases often present with non-specific systemic symptoms like rash and fever, which precede more specific symptoms like arthritis. Diagnosis of autoinflammatory diseases is often difficult due to the presentation with non-specific symptoms and the low incidence of these diseases. Patients are often empirically treated for more likely causes of symptoms, including infections. Such delay in diagnosis and initiation of appropriate treatment is suboptimal for the child and may also result in misuse of antibiotics, contributing to emergence of antibiotic resistance. To differentiate patients with auto-inflammatory diseases (e.g., systemic onset juvenile idiopathic arthritis) from patients with acute viral and bacterial infections, Allantez et al. analyzed leukocyte gene expression profiles of different blood leukocyte subpopulations that were obtained from these patients. Based on their results, a specific blood signature was developed that enabled differentiation between infection and other febrile inflammatory diseases.[41]

Along with permitting characterization of infections when pathogens are not directly detectable, measurement of specific gene expression by the host can potentially permit distinction between colonization and infection with pathogenic microorganisms. For example, secondary bacterial infections in children with viral lower respiratory tract infections are often diagnosed based on cultures from upper airway samples. The question remains whether the detected organism is really the cause of infection or whether it is just a contaminant from the upper airway.[42] The development of a diagnostic test based on gene expression patterns in the host may provide more specific information. In the future, the development of such diagnostic tools may help the clinician choosing an effective treatment strategy and reducing inappropriate antibiotic use.

A diagnostic delay in infectious diseases can lead to delayed initiation of therapy, severe complications, and long term consequences that may include death. Such a delay in diagnosis may be prevented by the development of diagnostic biomarkers. Encephalitis, for example, is a complex, severe, neurological syndrome associated with significant morbidity and mortality. It is characterized by inflammation of the brain parenchyma, and children often present with drowsiness, fever, headache, seizures, or focal neurological signs. A delay in

treatment may lead to irreversible brain damage. Diagnosis is often presumptive and based on clinical characteristics or increased serological antibody titers. Unfortunately, the causative pathogen is often hard to detect in the central nervous system itself. The final diagnosis is sometimes based on the detection of pathogens in cultures from other sites such as the respiratory tract.[43] Indirectly diagnosing encephalitis based on respiratory samples is rather inaccurate and demonstrates the need for new and better diagnostic tools. New microarray and proteomics technologies may contribute to improved diagnosis and treatment. Microarrays have been developed to simultaneously identify different viral and bacterial pathogens in cerebrospinal fluid (CSF).[44, 45] To our knowledge, gene expression studies for differentiating pathogens based on the host response have not yet been performed. The differentiation of pathogens based on the host response may provide better insight in pathogenesis and disease specific profiles in blood or cerebrospinal fluid; it may also prove useful in diagnosing encephalitis. The identification of biomarkers related to clinical profiles or recognition of subgroups in encephalitis may help predict outcome and provide insight into the efficacy of therapy.

Another infection in which diagnostic difficulties often lead to a delay in appropriate therapy is infective endocarditis. The clinical diagnosis of infective endocarditis may be difficult, as fever can be the only symptom. Rapid diagnosis followed by appropriate treatment is of critical importance for survival. However, in 3–31% of patients, causative pathogens remain undetected. Fenollar et al. analyzed serum samples from 88 patients with a clinical suspicion of endocarditis. They identified 66 different protein peaks in patients with confirmed endocarditis as compared to those in whom the diagnosis was excluded.[46] From these 66, they developed a diagnostic assay based on 7 protein peaks. Despite this limited number of differentially expressed proteins, the test was still able to classify the majority (88%) of patients correctly.

Future Perspectives for Biomarker Development

Proteomic and genomic technologies have been shown to contribute to improved insight into disease pathogenesis and may be useful in diagnosing infections and providing information about disease susceptibility. However, clinical application of these technologies has not yet been developed. Future research should focus on the validation of previously identified biomarkers as well as the development of new diagnostic assays.

The relationship between gene expression profiles and disease outcome is

another interesting field of research. Prognostic biomarkers could be important in infectious diseases, helping to predict disease outcome and to select patients that may benefit from treatment. At present, their clinical use is limited to the field of cancer research where several studies suggest that molecular classification of tumors, based on gene expression, may identify distinct prognostic subtypes. Alizedah et al. have detected two subtypes of diffuse B-cell lymphoma with different survival patterns.[47] The Mamma print, a test developed by the Dutch Cancer Institute, identifies different breast cancer subtypes by analysis of expression profiles involving 70 genes indicative of poor prognosis.[48, 49] These studies are based on hierarchical clustering of subgroups with similar gene expression profiles. Hierarchical clustering methods in gene expression proriling might prove useful in pediatric infectious diseases; although to date, few studies have been done. Chaussabel et al. have generated a potentially useful framework for the visualization and functional interpretation of microarray based disease specific transcriptional signatures.[50] In addition, the identification of biomarkers for monitoring inflammatory disease activity (in SLE) may contribute to better evaluation of disease progression and thus prognosis.[50]

At our department, we are currently conducting a clinical study to identify classifier genes that can assess disease severity in children suffering from viral LRTIs (VIRGO study). Using microarray analyses of blood leukocytes and respiratory samples, we aim to identify biomarkers that distinguish children with a relatively mild course of disease from those who will deteriorate and require supplemental oxygen or mechanical ventilation. In the early phase of infection, this may help decide whether a child needs to be hospitalized.

Another potential application is to guide treatment by allowing therapy to be tailored both to the specific pathogen, including its antimicrobial resistance properties and to host characteristics, including the immune response, which leads to more focused drug use and improved outcome. An early example of genotype guided, individualized treatment strategy is the adjustment of isoniazid dosing regimen in adults with tuberculosis. N-acetyltransferase type 2 (NAT2) plays an important role in isoniazid metabolism and genetic polymorphisms of this enzyme can alter the response to the drug. Determining the NAT2 genotype prior to isoniazid administration can predict individual pharmacokinetic variability and therapeutic response.[51]

Individualized treatment strategies will be extremely helpful in treating tuberculosis in children. At present, children with tuberculosis are treated with up to four tuberculostatic agents for two months followed by a two drug regimen during a 4 month continuation phase. To date, there is no laboratory

tool to monitor response to therapy. Moreover, difficulties in identification of *M. tuberculosis* from, for example, induced sputum or gastric lavage, contribute to diagnostic and therapeutic uncertainty.[52] Consequently, non-specific clinical features such as symptom improvement, weight gain, and radiological features of chest disease are used as markers for therapeutic response.[53] The identification of biomarkers for tuberculosis disease activity may provide more specific monitoring strategies leading to more focused prescribing, fewer adverse effects, and less multidrug resistance.

CONCLUSIONS

Diagnostic uncertainty in infectious disease may result in a delay in diagnosis and inappropriate use of antibiotics. The development of diagnostic biomarkers for infectious diseases may contribute to more rapid diagnosis, more reliable discrimination between infection and non-infectious diseases, more improved management, better course and outcomes, and less inappropriate use of antibiotics. Microarray and proteomic technologies are beginning to contribute to improved understanding of the pathogenesis of a wide variety of infectious diseases and have great prospects for the future. These technologies can be targeted both at direct pathogen detection and at characterization of the host response, which may also assist in diagnosis and disease monitoring as well as predicting the individual's susceptibility to disease, response to medical therapy, and overall prognosis. Although promising, the clinical application of these technologies in infectious diseases is limited at present. Current research focuses on sophisticated highly specialized techniques, but future work will need to be directed at clinical validation studies to collect data on clinical applicability, accuracy and cost effectiveness. Translating biomarker research into clinically useful tests will be difficult and time and labor intensive. The ultimate goal is to develop clinically relevant, cheap, rapid diagnostic and prognostic biomarker tests which use biological samples that are easy to obtain from the patient and which generate reliable and easily interpreted results.

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VIRAL FACTORS AND DISEASE SEVERITY

Chapter 3

Infection with multiple viruses is not associated with increased disease severity in children with bronchiolitis

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ABSTRACT

Background

The clinical relevance of parallel detection of multiple viruses by real-time polymerase chain reaction (RT-PCR) remains unclear. This study evaluated the association between the detection of multiple viruses by RT-PCR and disease severity in children with bronchiolitis.

Methods

Children less than 2 years of age with clinical symptoms of bronchiolitis were prospectively included during three winter seasons. Patients were categorized in three groups based on disease severity; mild (no supportive treatment), moderate (supplemental oxygen and/or nasogastric feeding), and severe (mechanical ventilation). Multiplex RT-PCR of 15 respiratory viruses was performed on nasopharyngeal aspirates.

Results

In total, 142 samples were obtained. Respiratory syncytial virus (RSV) was the most commonly detected virus (73%) followed by rhinovirus (RV) (30%). In 58 samples (41%) more than one virus was detected, of which 41% was a dual infection with RSV and RV. In RSV infected children younger than 3 months, disease severity was not associated with the number of detected viruses. Remarkably, in children older than 3 months we found an association between more severe disease and RSV mono-infections.

Conclusion

Disease severity in children with bronchiolitis is not associated with infection by multiple viruses. We conclude that other factors, such as age, contribute to disease severity to a larger extent.

INTRODUCTION

In young children, bronchiolitis is a common presentation of viral lower respiratory tract infections (LRTI). Human Respiratory syncytial virus (RSV) is the most frequently identified virus with detection rates up to 40–85% in infants hospitalized for respiratory infections during winter epidemics.[1, 2] About 1–2% of children infected by RSV need to be hospitalized, of which 6–11% require intensive care admission.[3, 4] Other viruses which are frequently detected in young children with acute respiratory tract infections include: rhinovirus (RV), human metapneumovirus (hMPV), parainfluenza virus (PIV), influenza virus (IV), adenovirus (AdV), enterovirus (EV) and human bocavirus (hBoV).[5–7]

Children with bronchiolitis show a great variability in disease severity. Although prematurity, congenital heart diseases, chronic lung disease, and immune deficiencies are known risk factors for severe RSV infection, half of the children admitted at an intensive care unit were born at term and healthy.[8] It is still not completely understood why some children develop a more severe course of disease than others. Both host and viral factors contribute to viral pathogenesis and disease severity is the result of a dynamic interplay between these factors.

The use of real-time polymerase chain reaction (RT-PCR) has greatly improved the ability to diagnose viral respiratory infections. PCR based methods are more sensitive than conventional detection methods such as viral culture and antigen detection. Furthermore, RT-PCR has enabled the identification of viruses which are normally difficult to detect by conventional methods and has allowed the simultaneous detection of multiple pathogens in one sample.[9]

As a result of the latter, the detection of viral co-infection in children with LRTIs has increased from 5 to 10% using conventional methods to 10–30%.[5, 10, 11] However, the clinical implications of these co-infections remain unclear. Some reports have suggested that infection with multiple viruses results in a more severe course of disease, while others have described that disease severity did not differ between infections caused by one or multiple viruses.[10, 12, 13] In addition, the presence of viruses in asymptomatic children suggests that a positive viral PCR does not necessarily indicate a causative relationship.[14]

In this study, we aim to examine whether infection with multiple viruses results in increased disease severity in young children with bronchiolitis. Therefore, we prospectively studied the association between the detection of multiple viral pathogens by RT-PCR and disease severity in young children with bronchiolitis included during three consecutive winter seasons.

METHODS

Patients

Children younger than 2 years of age with clinical symptoms of bronchiolitis presenting to the emergency department or the departments of pediatrics of the Radboud University Medical Center or Canisius Wilhelmina Hospital, Nijmegen, the Netherlands, were prospectively included between November and April (winter season) in the years 2006–2009. Bronchiolitis was defined as an acute infection of the lower airways, characterized by increased respiratory effort (tachypnea and/or use of accessory respiratory muscles) and expiratory wheezing and/or crackles and/or apnea. Medical history and demographic data were collected from questionnaires and medical records. The study was approved by the Committee on Research involving Human Subjects of the University Nijmegen Medical Centre and written informed consent was obtained from all parents. Within 24 hr after admission a nasopharyngeal aspirate was collected and stored at -80˚C for virological characterization. Patients were classified into three different groups based on the severity of disease. Children without hypoxia or need for nasogastric feeding were allocated to the mild group. The moderate group included children requiring hospitalization for supplemental oxygen and/or nasogastric feeding. Supplemental oxygen was given at oxygen saturations below 93% measured by pulsoximetry. Finally, children requiring mechanical ventilation were included in the severe group.

Collection of Nasopharyngeal Aspirates

A nasopharyngeal aspirate was collected by introducing a catheter, connected to a collection tube and aspiration system, through one of the nostrils into the nasopharyngeal cavity. Then, 1.5 ml of saline was instilled into the catheter and, while slowly retracting the catheter, the nasopharyngeal fluid was aspirated in a collection tube. Subsequently the catheter was flushed with 1 ml of saline and added to the collection fluid. The samples were cooled and transported to the laboratory. The nasopharyngeal aspirate was centrifuged at 500g for 10 min at 4˚C and the supernatant was frozen at -80˚C.

Virus Detection in Nasopharyngeal Secretions

Samples were analyzed by multiplex RT-PCR as previously described.[9] Briefly, upon

thawing, nucleic acids were extracted from each sample, using the MagNA Pure LC and the MagNA-Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Almere, The Netherlands) according to the instructions of the manufacturer. A multiplex RT-PCR assay containing 15 different viral pathogens was used. This assay was designed for the detection of specific viral genomes belonging to IV type A and B, coronavirus (CoV) 229E and OC43, hBoV, EV, AdV, parechovirus (PeV), PIV types 1–4, hMPV, RV, and RSV. An internal control consisting of Phocine Herpesvirus (PhPV, IC DNA control) and Equine Arthritis Virus (EAV, IC RNA control) was included in the assay. RNA was reverse transcribed to cDNA using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Nieuwerkerk Ad Ijssel, NL) in a 50 µl reaction mix containing 20 µl of nucleic acid isolate and random hexamers as primers, according to the manufacturer's instructions. PCRs were performed on the LightCycler 480 instrument using LightCycler 480 Probes Master Mix (Roche Diagnostics). Validated primer/probe-mixes were purchased from Diagenode (Liège, Belgium) and used according to the manufacturer's instructions. Cycling conditions were 95°C for 5 min, followed by 50 cycles of 95°C for 15 sec and 55°C for 15 sec and 72°C for 20 sec. The amount of virus was recorded semi-quantitatively based on the cycle threshold value (Ct value).

Statistical Analysis

Values are expressed as percentages for discrete/categorical variables and as median and interquartile range (IQR) for continuous variables. As the data were not normally distributed, Kruskal–Wallis tests were performed to compare age, birth weight, and symptoms duration. Mann–Whitney U-tests were used for individual comparisons between mild, moderate, and severe patients. Chi-squared tests were performed to compare categorical data. A two sided value of $p < 0.05$ was considered statistically significant.

RESULTS

One hundred and forty two children were included. Table 1 shows the characteristics of the patients. The mean age was 4.5 months. The age distribution was: 76 children younger than 3 months (54%), 25 between 3 and 6 months (18%), 27 between 6 and 12 months (19%), and 14 between 1 and 2 years (10%). Children in the severe group were younger than those in the mild and moderate group

(2.2 vs. 4.7 and 6.4 months, respectively; p<0.001). Twenty-seven children (19%) had underlying diseases, of which 18 (13%) were born prematurely (defined by a gestational age of 35 weeks or less) and 9 had a congenital heart defect (6%). Prematurity (27% vs. 6%; P=0.02) and maternal smoking during pregnancy (33% vs. 12%; P=0.01) was more often observed in the severe group compared to the moderate group. The lower day care attendance in the severe group can be explained by the lower age in this group. No other significant differences in clinical parameters were found between the patient groups.

Overall, 211 viruses were detected in 142 nasopharyngeal aspirates. Data are summarized in Table 2. In 4 of the 142 samples (3%) no virus was detected. The most frequently detected virus was RSV in 104 samples (73%), followed by RV, which was present in 43 samples (30%).

Table 1. Patient characteristics

	Total (N=142)	**Mild (N=41)**	**Moderate (N=64)**	**Severe (N=37)**	**P-value**
Age (mo), mean±SE	4.5±0.39	6.4±0.81	4.7±0.61	2.2±0.36	p<0.001[3]
Male	87 (61)	23 (56)	38 (59)	26 (70)	NS
Birth characteristics					
Birth weight (g), mean±SE	3261±66	3202±123	3405±91	3094±135	NS
Prematurity[1]	18 (13)	4 (10)	4 (6)	10 (27)	p=0.01[4]
Maternal smoking	22/130 (17)	5/39 (13)	7/61 (12)	10/30 (33)	p=0.03[5]
Breastfeeding	81/138 (59)	28/41 (68)	34/61 (56)	19/36 (53)	NS
Underlying diseases[2]					
CHD	9 (6)	3 (7)	2 (3)	4 (11)	NS
Environmental factors					
Atopic disease	18/140 (13)	6 (15)	9 (14)	3/35 (9)	NS
Atopic family history	76/136 (56)	19/40 (48)	38/62 (61)	19/34 (56)	NS
Passive smoking	19/134 (14)	6/39 (15)	7/63 (11)	6/32 (19)	NS
Siblings	110/141 (78)	30 (73)	47/63 (75)	33 (89)	NS
Daycare attendance	25/138 (18)	10/38 (26)	14/64 (22)	1/36 (3)	p=0.02[6]
Presentation					
Days of illness before presentation, median (IQR)	4.0 (3.0–6.0)	4.0 (3.0–7.5)	4.0 (3.0–6.0)	5 (3.0–6.0)	NS

Data are presented as number (%), unless otherwise specified. Mo, months; g, grams; IQR, interquartile range; CHD, congenital heart defect. [1]prematurity was defined as a gestational age of 35 weeks or less.[2]no patients with chronic lung diseases or immune deficiencies were included. Individual statistical comparisons were as follows: [3]mild versus moderate, p=0.03; mild versus severe, p<0.001; moderate versus severe, p=0.01. [4]moderate versus severe, p= p0.02. [5]moderate versus severe, p=0.01. [6]mild versus severe, p=0.001; moderate versus severe, p=0.002.

Table 2. Distribution of viruses recovered from 142 nasopharyngeal aspirates from children less than 2 years of age with bronchiolitis

Virus	Total (N=142)	Mild (N=41)	Moderate (N=63)	Severe (N=37)
RSV	104 (73)	29 (71)	47 (75)	28 (76)
RV	43 (30)	17 (41)	20 (32)	6 (16)
AdV	13 (9)	2 (5)	7 (11)	4 (11)
EV	10 (7)	5 (12)	5 (8)	0
hMPV	9 (6)	4 (10)	4 (6)	1 (3)
IV-A	7 (5)	1 (2)	5 (8)	1 (3)
hBoV	6 (4)	4 (10)	2 (3)	0 (0)
CoV	8 (6)	5 (12)	2(3)	1 (3)
PIV-3	6 (4)	2 (5)	4 (6)	0
PeV	5 (4)	1 (2)	4 (6)	0
No virus	4 (3)	0	0	4 (11)
1 virus	80 (56)	18 (44)	35 (56)	26 (70)
> 1 virus	58 (41)	23 (56)	28 (44)	7 (19)
2 viruses	46 (32)	18 (44)	22 (35)	6 (16)
3 viruses	10 (7)	3 (7)	6 (10)	1 (3)
≥ 4 viruses	2 (1)	2 (5)	0	0

Data are presented as number (% of samples). Total exceeds 100% because of the detection of more than one virus per sample. RSV, respiratory syncytial virus; RV, rhinovirus; AdV, adenovirus; EV, enterovirus; hMPV, human metapneumovirus; IV, Influenzavirus; hBoV, human bocavirus; CoV, coronavirus; PIV, parainfluenzavirus; PeV, parechovirus. IV-B, PIV type 1,2, and 4 were not detected and not mentioned in this table.

Other respiratory viruses were found in less than 10% of the total group. RSV was detected in a similar frequency in the three severity groups (71–75%), while RV was found in 41, 32, and 16% of children with mild, moderate, and severe disease, respectively.

More than one virus was found in 58 of 142 subjects (41%). Figure 1 shows the distribution of viruses detected as a single infection or in combination with other viruses. RSV was detected as a single virus infection in 61 of RSV positive subjects (59%), followed by hMPV which was detected as a single virus in respectively 56% of hMPV positive samples. RV was detected as a single virus in 9 of 43 subjects with RV positive samples (21%). The other viruses were less frequently detected as single virus infections, of which hBoV, PeV, and AdV were only detected in combination with other viruses.

Infection with two or more viruses was more frequently found in children with mild and moderate disease than in those with severe disease (56 and 44%, respectively vs. 19%; $p=0.003$). Infection with both RSV and RV was the most

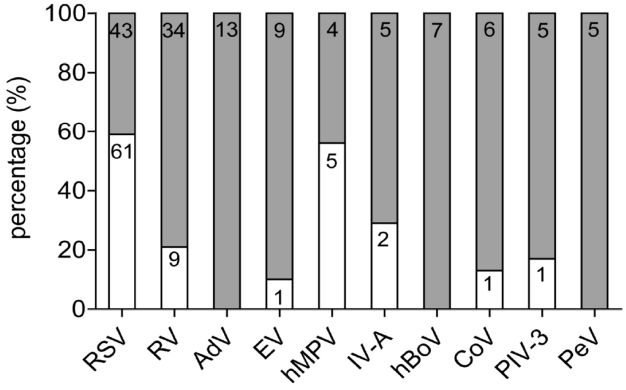

Figure 1. Frequencies of viruses detected as a single virus or in combination with other viruses. Numbers in bars represent the absolute numbers of infection per virus. RSV, respiratory syncytial virus; RV, rhinovirus; AdV, adenovirus; EV, enterovirus; hMPV, human metapneumovirus; IV, influenza virus; hBoV, human bocavirus; CoV, coronavirus; PIV, para-influenza virus; PeV, parechovirus.

common combination of viruses, detected in 24 of the 58 infections caused by multiple viruses (41%), followed by the combination of Adv and RSV in 9 patients (16%).

Table 3 depicts the clinical characteristics and disease severity in children with either RSV mono- and multiple infections or children infected by one or more other viruses than RSV. Children with RSV mono-infections were younger (2.0 months vs. 5.6 and 5.4 months; p=0.001) and suffered from more severe disease than children with RSV multiple infections and children infected with other viruses than RSV. Children with RSV mono infections required more often mechanical ventilation compared to those with RSV and one or more other viruses (36% vs. 14%; p=0.002). There was a trend toward lower Ct values, implicating higher viral load, in more severe disease in children with RSV multiple infections, but not in children with RSV mono-infections.

As the differences in age between the groups may have influenced our results, we also evaluated the association between disease severity and the detection of multiple viruses in children diagnosed with RSV bronchiolitis younger and older than 3 months (Fig. 2). Prematurity was more often observed in RSV infected children older than 3 months than in those below 3 months. Other risk factors were not different between these two age groups. Children younger than 3 months were less often infected by multiple viruses compared to children older than 3 months (25% vs. 65%). In children younger than 3 months disease severity was not associated with the number of detected viruses, while in children older

than 3 months significantly less multiple infections were detected in the severe group compared to the mild group (33% vs. 84%; p<0.01).

Table 3. Comparison of patient characteristics and disease severity in patients with RSV mono-infections, multiple infections including RSV and infections caused by other viral pathogens

	RSV mono (N=61)	RSV multiple (N=43)	RSV negative (N=38)	P-values
Age (mo), median (IQR)	2.0±0.39	5.6±0.84	5.4±0.80	p<0.001[3]
Age<3 mo	46 (75%)	15 (35%)	15 (39%)	p<0.001[3]
Male	41 (67%)	25 (58%)	21 (55%)	
Birth characteristics				
Birth weight (grams), mean±SD	3251±778	3281±700	3255±817	
Prematurity[1]	9 (15%)	6 (14%)	3 (8%)	
Maternal smoking	12/59 (20%)	6/40 (15%)	4/31 (13%)	
Breastfeeding	33/60 (53%)	29/41 (71%)	20/37 (54%)	
Underlying diseases[2]				
CHD	1 (2%)	2 (5%)	5 (13%)	p=0.03[4]
Environmental factors				
Atopic disease	6 (10%)	6/42 (14%)	6/37 (16%)	
Atopic family history	34/59 (58%)	19/42 (45%)	23/35 (66%)	
Passive smoking	9/59 (15%)	4/42 (10%)	6/33 (18%)	
Siblings	50 (82%)	33 (77%)	27/37 (73%)	
Daycare attendance	1 (2%)	13/41 (32%)	11/36 (31%)	p<0.001[3]
Viral load				
RSV Ct value, mean±SD	27.2±5.1	28.3±4.4		
Mild	26.5±7.2	29.2±4.9		
Moderate	27.2±4.8	28.1±3.7		
Severe	27.5±4.3	26.0±3.4		
Disease severity				p=0.002[5]
Mild	11 (18%)	18 (42%)	12 (32%)	
Moderate	28 (46%)	19 (44%)	17 (45%)	
Severe	22 (36%)	6 (14%)	9 (24%)	

Data are presented as number (%), unless otherwise specified. IQR, interquartile range; SD, standard deviation; CHD, congenital heart defect; mo, months. [1]prematurity was defined as a gestation age of 35 weeks or less. [2]no patients with chronic lung diseases or immune deficiencies were included. [3]RSV negative versus RSV mono and RSV negative versus RSV multiple. [4]RSV negative versus RSV mono. [5]RSV multiple versus RSV mono.

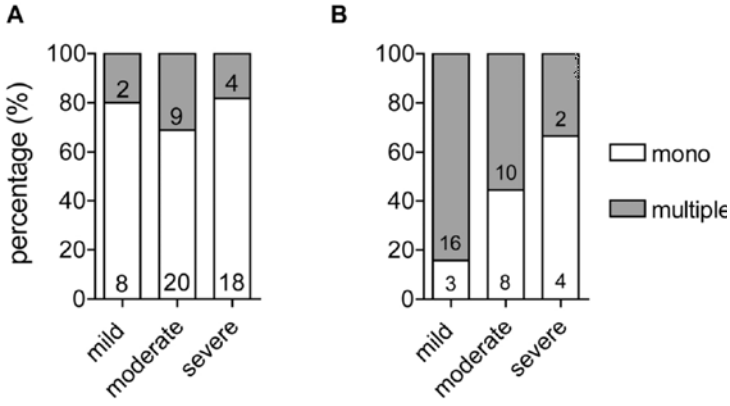

Figure 2. Percentages of infections caused by RSV (mono) or RSV and ≥1 other viruses (multiple) in children (A) younger and (B) older than 3 months of age for the different severity groups. Numbers in bars are absolute numbers.

DISCUSSION

In the present study, we evaluated the viral etiology in young children with bronchiolitis during three consecutive winter seasons and examined the association between the detection of two or more viruses by RT-PCR and disease severity. Our main finding was that the detection of more than one virus is not associated with increased disease severity in children with bronchiolitis. These findings debate the cumulative effect of the detection of a certain virus on disease severity during co-infection. In addition, viral load in children with single RSV infections is not associated with disease severity.

The amount of multiple infections (41%) in this study is consistent with current literature.[5, 10, 11] However, in 97% of our samples at least one virus was detected which is high compared to other studies.[15, 16] This may be explained by the inclusion period between November and April in which virus activity as reflected in the number of respiratory tract infections is highest in The Netherlands in combination with strict inclusion criteria of bronchiolitis. Furthermore, RT-PCR is a sensitive method to detect viruses. As the detection of a virus by RT-PCR does not necessarily mean that it causes symptoms, the presence of a virus, RV particularly, has to be interpreted carefully. Viral RNA is reported to be detectable in nasopharyngeal samples 4–5 weeks after infection.[17, 18] RV has been detected in 12–35% of asymptomatic children,[19–21] while asymptomatic carriage of other viruses, especially RSV, is uncommon with detection rates up to 5% only.[7,14,18,21,22]

In this study, infection with both RSV and RV was the most common combination of viral co-infections. To date there has been little agreement on the effect of viral co-infection on disease severity in children with bronchiolitis in which both RSV and RV are detected. Papadopoulos et al. demonstrated that the presence of RV in children with RSV bronchiolitis increased the risk for severe disease approximately five times.[13] Although this study was supported by another study that also described dual viral infection as a relevant risk factor for intensive care admission[23], others did not find an additional effect of RSV/RV dual infections on disease severity compared to RSV single infections.[15, 24]

RSV has been described as the most common pathogen causing bronchiolitis and is associated with increased disease severity in young children and in those with underlying diseases.[24, 25] In our study, children with RSV mono-infections suffered from most severe disease. The young age of the children in the most severe group may have caused a bias in our results, since young age is a well-known risk factor for severe RSV infection. This is supported by our findings that demonstrate that children with severe symptoms upon viral infection are younger than children with milder manifestations of viral infection. Correction for age is difficult, as age, disease severity, and multiple infections are all related to each other. To correct for age we performed analyses in children younger and older than 3 months.

In children younger than 3 months no association was found between disease severity and the number of detected viruses. The higher prevalence of prematurity in children older than 3 months compared to those below 3 months suggest that the youngest children are most susceptible for severe disease upon viral infection and that in older children other risk factors, such as prematurity, become more important contributors to disease severity.

Interestingly, in children older than 3 months, an association between severe disease and the presence of one virus was observed (Fig. 2). In line with this finding, Marguet et al. observed a shorter duration of hospitalization in children with RSV/RV dual infection compared to those with single RSV infection suggesting that infection with RV has no additional, and potentially a protective effect on disease severity.[12] In addition, children with multiple viral infections including RSV did not suffer from more severe disease compared to those without RSV. This may be explained by either differences in age or type of immune response. The induced immune response upon viral infection may protect the host from infection with a second virus as has been proposed by Greer et al. who described a potential protective effect of RV through the stimulation of the interferon (IFN) stimulated genes inducing an antiviral state that prevents the patient from a

more severe course of disease after second infection with a new virus.[26]
We also observed an association between age and multiple infections. Less multiple infections were observed in younger children. This has been described before[11] and possible explanations why detection of more than one virus is less frequently observed in the most youngest infants are; (1) less exposure to viruses due to less day-care attendance, (2) the development of more and earlier severe symptoms upon single viral infection, and (3) partial protection against respiratory viruses because of protective maternal antibodies which disappear with age.
In addition to host factors, such as young age and underlying diseases, type of virus and viral load have been described to play a role in disease severity.[27, 28] In our study, a trend toward higher RSV loads in children with more severe disease was observed in children with RSV multiple infection but not in those with RSV mono-infections. Most studies have described a positive relation between higher RSV load and disease severity.[28–31] In addition, an association of higher viral load with young age has been previously reported.[32] Our sample size was rather small compared to these studies and age specific analyses for viral loads were therefore not performed. Finally, a number of limitations need to be considered. First, we only included children during the winter season, from November until April. This may have created a bias toward a higher incidence of RSV. While RV infections are present throughout the year with peaks in autumn and spring, RSV does rarely appear out of winter season in the Netherlands. Data from a registry of the Dutch Working Group on Clinical Virology showed that in 2006 and 2007 51% of the annual RV infections were detected between November until April compared to 94% of the annual RSV infections (published with permission of the Dutch Working Group on Clinical Virology). Second, we only included children presenting in a hospital, which may have caused a bias. Third, we analyzed infections with other viruses than RSV as a group (RSV negative infections) instead of analyzing them separately, because the low number of samples infected with other viruses than RSV. To further reveal the association between young age, multiple infections and disease severity in children with bronchiolitis, more large-scale clinical studies in various health care settings are required.
To conclude, in this study we showed that disease severity in children with bronchiolitis is not associated with infection by multiple viruses. Remarkably, in children older than 3 months we found an association between more severe disease and RSV mono-infections. Our results suggest that other factors than infection with multiple viruses contributes to disease severity, of which age is the most important risk factor.

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HOST IMMUNE RESPONSE AND DISEASE SEVERITY

Chapter 4

Use of MMP-8 and MMP-9 to assess disease severity in children with viral lower respiratory tract infections

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ABSTRACT

Introduction

Matrix metalloproteinases (MMPs) play an important role in respiratory inflammatory diseases, such as asthma and chronic obstructive pulmonary disease. It was hypothesized that MMP-8 and MMP-9 may function as biological markers to assess disease severity in viral lower respiratory tract infections in children.

Methods

MMP-8 and MMP-9 mRNA expression levels in peripheral blood mononuclear cells (PBMCs) and granulocytes obtained in both the acute and recovery phase from 153 children with mild, moderate, and severe viral lower respiratory tract infections were determined using real-time PCR. In addition, MMP-8 and MMP-9 concentrations in blood and nasopharyngeal specimens were determined during acute mild, moderate, and severe infection, and after recovery using ELISA. Furthermore, PBMCs and neutrophils obtained from healthy volunteers were stimulated with RSV, LPS (TLR4 agonist), and Pam3Cys (TLR2 agonist) in vitro.

Results

Disease severity of viral lower respiratory tract infections in children is associated with increased expression levels of the MMP-8 and MMP-9 genes in both PBMCs and granulocytes. On the contrary, in vitro experiments showed that MMP-8 and MMP-9 mRNA and protein expression in PBMCs and granulocytes is not induced by stimulation with RSV, the most frequent detected virus in young children with viral lower respiratory tract infections.

Conclusion

These data indicate that expression levels of the MMP-8 and MMP-9 genes in both PBMCs and neutrophils are associated with viral lower respiratory tract infections disease severity. These observations justify future validation in independent prospective study cohorts of the usefulness of MMP-8 and MMP-9 as potential markers for disease severity in viral respiratory infections.

INTRODUCTION

Respiratory viral infections are an important cause of hospitalization among children younger than 5 years of age with estimated population-based hospitalization rates of 1–2%.[1-3] Human respiratory syncytial virus (RSV) is the most commonly identified virus with detection rates up to 40–85% in infants hospitalized for respiratory infections during winter epidemics.[4-6] The clinical manifestations range from a simple common cold to severe lower respiratory tract symptoms requiring mechanical ventilation. About 6–11% of the children admitted to hospital with RSV infection require intensive care admission.[7, 8] Up to 35% of the children hospitalized with bronchiolitis did not receive any supportive intervention.[9] On the other hand, it is crucial to avoid discharge of those children who may experience clinical deterioration. Among children sent home with the diagnosis bronchiolitis, 4.6–6.8% required hospitalization later on during infection.[10, 11] Biomarkers to assess severity of viral lower respiratory tract infections (LRTIs), in particular RSV infection, may be helpful to clinicians in the decision whether a child needs to be hospitalized.

Lung injury during severe RSV infection is thought to be mediated by both direct cytotoxic effects of the virus and the result of the induced inflammation. Pathologic features of severe RSV infection include extensive bronchiolar epithelial destruction, peribronchial lymphocyte infiltration, necrosis of bronchial epithelium, and mucus plugs in the small bronchioles.[12, 13] Matrix metalloproteinases (MMPs) are family of zinc endopeptidases capable of degrading components of the cellular matrix, and consequently, are suggested to be important in several diseases associated with tissue remodeling. Pronounced increase in their expression is thought to be associated with a variety of inflammatory disease, including respiratory diseases.[14]

MMPs play a role in cellular migration of neutrophils, lymphocytes, and other immune cells to the lungs by degrading extracellular matrix, but also have pro- and anti-inflammatory properties. The activity of MMPs is regulated through binding to tissue inhibitor of metalloproteinases (TIMPs) leading to inactivation. An imbalance in production and activation, or inactivation by TIMPs might augment airway inflammation through direct or indirect effects upon signaling pathways that influence migration of leukocytes through the tissues.[14,15] Increased concentrations and activity of MMP-8 and MMP-9 have been observed in respiratory samples obtained from adults and children with acute lung injury and pneumonia[16-19] as well as in chronic lung diseases such as asthma.[20, 21] In addition, a relation between MMP-9 concentrations and disease severity of

pneumonia[16, 18] and asthma[22, 23] has been described.
Yeo et al. have reported that MMP-9 protein expression is increased in human airway epithelial cell lines infected with RSV.[24] In addition, MMP-9 gene expression is increased in the lungs of RSV-infected mice.[25] Another study demonstrated that nasopharyngeal samples from infants infected with RSV and parainfluenza virus (PIV) contain increased MMP-9 and TIMP-1 concentrations.[26]
In the current study, it was hypothesized that MMP-8 and MMP-9 gene expression levels, and consequently, MMP-8 and MMP-9 plasma concentrations may function as biomarkers for disease severity in viral LRTIs.

METHODS

Study Design

Children younger than 5 years of age with laboratory confirmed viral LRTIs were prospectively included during three consecutive winter seasons (November–April in the years 2006–2009). Patients with congenital heart or lung disease, known immunodeficiencies or glucocorticoid use were excluded. Viral LRTI was defined as an acute infection of the lower airways, characterized by increased respiratory effort (tachypnea and/or use of accessory respiratory muscles and/or expiratory wheezing and/or crackles and/or apnea) in combination with a confirmed viral etiology by multiplex real-time polymerase chain reaction (RT-PCR) on nasopharyngeal washes as described previously.[27] The multiplex RT-PCR assay detect 15 different viral pathogens; influenza virus types A and B, coronavirus 229E and OC43, human bocavirus, enterovirus, adenovirus, parechovirus, PIV types 1–4, human metapneumovirus, rhinovirus (RV), and RSV.
Written informed consent was obtained from all parents and the study was approved by the Committee on Research involving Human Subjects of the University Nijmegen Medical Centre. Within 24 hr after admission a blood sample and nasopharyngeal aspirate was collected and parents from hospitalized children were asked permission to draw a second blood sample and nasopharyngeal aspirate 4–6 weeks after admission. Medical history, demographics, and clinical parameters were collected from questionnaires and medical records. Patients were classified into three different groups based on severity of disease. Children without hypoxia or severe feeding problems were allocated in the mild group, those requiring hospitalization for supplemental oxygen (oxygen saturations <93%) and/or nasogastric feeding in the moderate group and children requiring mechanical ventilation in the severe group.

Sample Collection

A nasopharyngeal aspirate was collected by introducing a catheter, connected to a collection tube and an aspiration system, into the nasopharyngeal cavity. Then, 1.5 ml of saline was instilled into the catheter and, while slowly retracting the catheter, the nasopharyngeal fluid was aspirated in a collection tube. Afterwards the catheter was flushed with 1 ml of saline and added to the collection fluid. The samples were kept cold and immediately transferred to the laboratory. The nasopharyngeal aspirate was centrifuged at 500g for 10 min at 4˚C to spin down the mucus and cells, after which the supernatant was frozen at -80˚C.
Five milliliters of blood was collected into sodium heparin tubes and directly transferred to the laboratory. A thin blood smear was prepared and stained with (May-Grunwald-)Giemsa to determine the percentages of granulocytes and PBMCs. PBMCs were obtained by density gradient centrifugation (Lymphoprep®; Axis Shield, Oslo, Norway) and stored in Trizol at -80˚C for RNA isolation. Plasma samples were stored at -80˚C for enzyme-linked immunosorbent assays (ELISAs).

Quantitative mRNA Expression of MMP-8 and MMP-9 in PBMCs and Granulocytes

RNA from PBMC and granulocytes was extracted using Trizol (Invitrogen Life Technologies, Bleiswijk, The Netherlands) according to the manufacturers' protocol. Subsequently, a clean-up was performed on total RNA with the RNeasy Minikit (Qiagen, Venlo, The Netherlands) according to the manufacturers' instructions. Total RNA (2 µg, measured with spectrophotometry, Nanodrop, Wilmington) was reverse transcribed using a high-capacity cDNA reverse transcription kit according to the manufacturers' instructions (Applied Biosystems, Foster City, CA) and cDNA was stored at -20˚. The relative gene expression was measured with SYBR Green PCR Mastermix (Applied Biosystems; P/N 4367659) on the ABI 7500 Fast Real Time PCR system using standard program and software. After 40 repetitions a dissociation curve was performed as control for the specificity of the PCR reaction. The following primers were used: hActin F: cgtcacacttcatgatggagttg, hActin R: cttccttcctgggcatgga; hMMP-9 F: gccccccttgcataagga, hMMP-9 R: cagggcgaggaccatagag; and hMMP-8 F: ccagtttgacatttgatgctatcac, hMMP-8 R: ctgaggatgccttctccagaa. All reactions were performed in duplo. Actin was used as reference gene. After a quality check (melting temp, curve of reaction, and standard deviation Ct) the ΔCt of the MMP-8 and MMP-9 to actin was calculated and expressed as relative expression.

MMP-8, MMP-9, and TIMP-1 Concentrations in Plasma and Nasopharyngeal Washes

Concentrations of total MMP-8 and MMP-9 in plasma, nasopharyngeal aspirate, and supernatants of cell stimulation assay were measured by ELISA according to the manufacturers' protocol (DuoSet, R&D systems, Abingdon, UK). In addition, TIMP-1 concentrations in plasma were determined as described above.

In Vitro Stimulation of PBMCs and Neutrophils From Healthy Volunteers

After informed consent, blood was drawn from healthy volunteers and collected in EDTA tubes. Blood was diluted 1:1 with pyrogene-free phosphate buffered saline (PBS) (Lonza, Basel, Switzerland). PBMCs and granulocytes were obtained by density gradient centrifugation (Lymphoprep®; Axis Shield). After washing, PBMCs were brought at a concentration of 5×10^6 cells/ml in serum-free RPMI medium (Gibco, Invitrogen, Paisley, UK) with 100 U/ml of penicilin/streptavidin (Gibco, Invitrogen). Granulocytes were purified by lysing the red blood cells (0.155 M NH4Cl, 0.0001 M Na2EDTA and 0.01 M KHCO3), and, after washing, granulocytes were suspended at a concentration of 5×10^6 cells/ml in RPMI medium supplemented with 0.5% human serum albumin (Sanquin, Amsterdam, The Netherlands).
Mononuclear cells (5×10^5 cells in 100 µl) were added to round-bottom 96-well plates and stimulated with either 100 µl culture medium (negative control), 1 ng/ml lipopolysacharides (LPS, *Escherichia coli* serotype 055:B5, Sigma–Aldrich, purified as described previously [28] or MOI 1 of RSV A2 (kindly provided by Dr. R. de Swart, Erasmus MC, Rotterdam, The Netherlands). RSV A2 was cultured in HeLa cells and purified by ultracentrifuge over a sucrose 30% gradient. After incubation for 24 hr at 37°C and 5% CO2. supernatant was collected and stored at -80°C. Neutrophils (5×10^5 cells in 100 µl) were stimulated and incubated in the same way for 4 hr and supernatant was stored at -80°C. Apoptosis was determined on the FACScalibur by Annexin V apoptosis detection kit (BD) according to the manufacturers' instructions and no differences between stimuli were found after 4 hr.

Statistics

Values are expressed as percentages for categorical variables and as mean and standard error (SE) or median and interquartile range (IQR) for continuous

variables. For variables that were not normally distributed, Kruskal–Wallis test was performed to compare continuous variables followed by Mann–Whitney U-tests for individual comparisons. Chi-squared tests were performed to compare categorical data. A two-sided value of $p<0.05$ was considered statistically significant.

RESULTS

Patient Characteristics

In total, 153 patients were included. In 109 patients (71%) RSV was detected. RSV positive children were significantly younger than RSV negative children. No other significant differences were observed between these groups (Table 1).

Table 1. Patient characteristics

	Total (N=153)	**RSV+ (N=109)**	**RSV- (N=44)**	**p-value**
Age (days±SE)	206±26	149±20	347±72	<0.001
Male	95 (62%)	70 (64%)	25 (57%)	NS
Prematurity	21 (14%)	16 (15%)	5 (11%)	NS
Family history of atopy	82 (57%)	56 (54%)	26 (63%)	NS
Symptomatic days before presentation (days±SE)	5.5±0.4	5.2±0.4	6.3±1.1	NS

Data are presented as number (%), unless otherwise specified. For variables that were not normally distributed, Kruskal–Wallis test was performed to compare continuous variables followed by Mann–Whitney U-tests for individual comparisons. Chi-squared tests were performed to compare categorical data. A two-sided value of $p<0.05$ was considered statistically significant. NS, not significant, SE, standard error.

A total of 54, 60, and 39 children were classified as having mild, moderate, and severe disease, respectively. Patients with severe disease were significantly younger compared to those with mild disease (105 days vs. 278 days; $p<0.05$). More prematurely born children were observed in the severe group compared to the mild and moderate group. No other significant differences in clinical parameters were found between the different severity groups (Table 2). In addition, total leukocytes and neutrophil counts were comparable between all groups.

Table 2. Patient characteristics for mild, moderate, and severe infections

	Total (N=153)	Mild (N=54)	Moderate (N=60)	Severe (N=39)	p-value
Age (days)	206±26	278±52	206±38	105±36	<0.01
Male (%)	95 (62%)	32 (59%)	36 (60%)	27 (69%)	NS
Prematurity (%)	21 (14%)	5 (9%)	6 (10%)	10 (26%)	<0.05
Family history of atopy (%)	82 (57%)	27 (52%)	36 (62%)	19 (55%)	NS
Symptomatic days before presentation	5.5±0.4	7.0±1.1	4.8±0.3	4.6±0.4	NS
RSV (%)	109 (71%)	35 (65%)	45 (75%)	29 (74%)	NS
Leukocytes counts	8.7±0.7	9.8±1.1	8.6±1.0	8.2±1.4	NS
Neutrophil counts	3.5±0.5	3.1±0.7	2.9±0.7	4.3±1.1	NS

Data are presented as percentages or mean ± standard error (SE). Cell counts are given as 10^6 cells/ml ±SE. For variables that were not normally distributed, Kruskal–Wallis test was performed to compare continuous variables followed by Mann–Whitney U-tests for individual comparisons. Chi-squared tests were performed to compare categorical data. A two-sided value of $p<0.05$ was considered statistically significant. NS, not significant.

Disease Severity Is Associated With Increased Gene Expression Levels of MMP-8 and MMP-9 in Both Granulocytes and PBMCs

During acute viral infection we observed increased expression of the MMP-8 and MMP-9 genes in both PBMCs and granulocytes compared to recovery. No differences in gene expression of MMP-8 and MMP-9 in both PBMCs and granulocytes were found between RSV positive and RSV negative children during acute infection. In general, gene expression of the MMP-9 gene was higher in granulocytes than in PBMCs. For MMP-8, the same trend was noticed (Figure 1A and B and figure 2A and B).

Increased disease severity was associated with higher expression levels of the MMP-8 and MMP-9 genes in both PBMCs and granulocytes. To determine whether this association was dependent on the type of virus, RSV positive and RSV negative children were analyzed separately. For RSV positive patients, the same association was found between disease severity and gene expression levels (Figs. 1C, D and 2 C, D). For RSV negative patients, MMP-8 and MMP-9 gene expression was higher in children with severe disease compared to those with mild. In addition, RSV negative children with severe disease had higher expression levels of the MMP-8 gene in both PBMCs and granulocytes compared to those with moderate disease (data not shown).

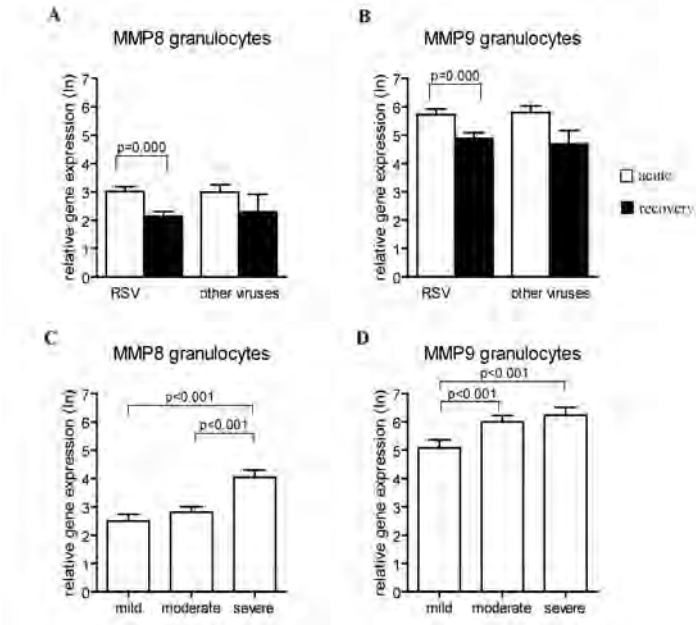

Figure 1. Gene expression levels of MMP-8 and MMP-9 in granulocytes from children with viral LRTIs. Relative gene expression levels (mean ± se) of MMP-8 (A) and MMP-9 (B) in granulocytes from children during acute RSV positive and RSV negative viral LRTIs and after recovery of infection. Relative gene expression levels of MMP-8 (C) and MMP-9 (D) for RSV positive children with mild, moderate, and severe disease. Mann–Whitney U-test was performed to compare children infected by RSV and other viruses. Paired analyses (Wilcoxon) were performed to compare acute and recovery samples. A two-sided value of $P < 0.05$ was considered statistically significant.

Disease Severity Is Associated With Increased MMP-8 Plasma Levels

The plasma concentration of MMP-8 was increased during acute RSV infection compared to recovery. In RSV negative patients this difference was not significant (Fig. 3A). Higher MMP-8 plasma concentrations were found in children with severe and moderate disease compared to those with mild disease (Fig. 4A). In nasopharyngeal washes, the concentration of MMP-8 was increased during acute RSV infection compared to recovery washes (Fig. 3D). No significant differences of MMP-8 concentrations in the nasopharyngeal washes were observed between the different severity groups (Fig. 4D). In both RSV positive as RSV negative patients MMP-9 concentrations in the nasopharyngeal washes were increased during infection (Fig. 3E). Children with moderate disease had increased MMP-9 concentrations in nasopharyngeal washes compared to those with mild disease.

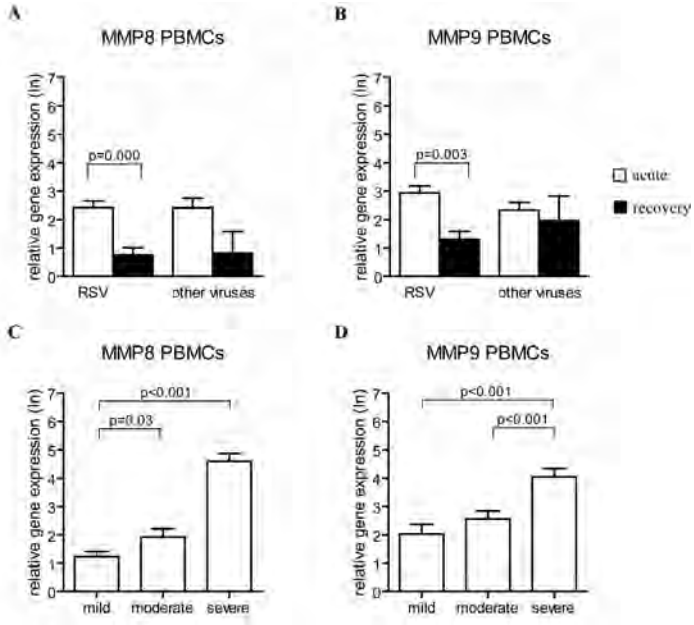

Figure 2. Expression levels of the MMP-8 and MMP-9 genes in PBMCs from children with viral LRTIs. Relative gene expression levels (mean ± standard error) of MMP-8 (A) and MMP-9 (B) in PBMCs from children during acute RSV positive and RSV negative viral LRTIs and after recovery of infection. Relative gene expression levels of MMP-8 (C) and MMP-9 (D) for RSV positive children with mild, moderate, and severe disease. Mann–Whitney U-test was performed to compare children infected by RSV and other viruses. Paired analyses (Wilcoxon) were performed to compare acute and recovery samples. A two-sided value of $p<0.05$ was considered statistically significant.

However, no significant differences in MMP-9 concentrations were observed in children with severe disease compared to those with mild and moderate disease (Fig. 4E). TIMP-1 concentrations in plasma or nasopharyngeal washes were not increased during acute infection and there was no correlation with disease severity (Figs. 3C and 4C). The ratio between MMP-9 and TIMP-1 plasma concentrations, an indicator for enzyme activity, was not increased during acute RSV infection. This ratio was significant higher in the recovery plasma of RSV negative patients compared to the acute samples (Fig. 3F). No relation between disease severity and the plasma MMP-9 and TIMP-1 ratio was found (Fig. 4F).
No differences in plasma or nasopharyngeal levels of MMP-8, MMP-9, and TIMP-1 plasma concentrations were observed between RSV positive and RSV negative children during acute viral respiratory infection. In general, MMP-8 and MMP-9 concentrations were higher in nasopharyngeal samples compared to plasma.

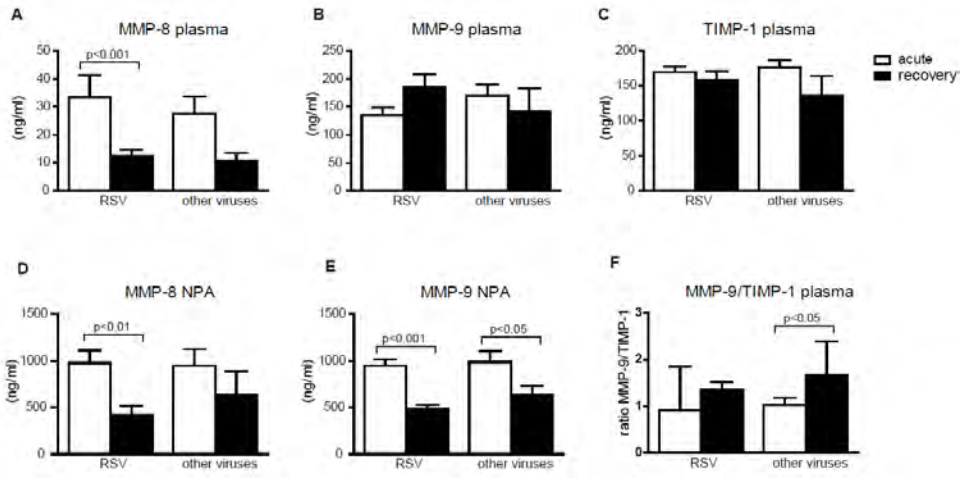

Figure 3. MMP-8 and MMP-9 concentrations in plasma and nasopharyngeal samples from children during acute LRTIs and after recovery. Plasma concentration of MMP-8 (A), MMP-9 (B), TIMP-1 (C), and concentration in nasopharyngeal washes of MMP-8 (D) and MMP-9 (E) from children during acute RSV positive and RSV negative viral LRTIs and after recovery of infection. Ratio's between plasma MMP-9 and TIMP-1 (F). Concentrations (ng/ml) are given in mean ± se. Mann–Whitney U-test was performed to compare children infected by RSV and other viruses. Paired analyses (Wilcoxon) were performed to compare acute and recovery samples. A two-sided value of $p<0.05$ was considered statistically significant.

MMP-9 Plasma Concentrations Are Correlated With the Number of Granulocytes

MMP-9 plasma concentrations correlated with the number of granulocytes measured during acute RSV infection (Pearson's correlation coefficient 0.33; P=0.019). No correlation was found between the number of granulocytes and MMP-8 plasma and nasopharyngeal concentrations and MMP-9 nasopharyngeal concentrations (data not shown). Furthermore, there was no correlation between symptomatic days before presentation and levels of MMP-8 and MMP-9 gene expression or concentration of the protein in plasma and nasopharyngeal washes (data not shown).

MMP-8 and MMP-9 mRNA and Protein Expression by PBMCs and Neutrophils Is Not Induced by RSV In Vitro

To investigate whether the source of plasma MMP-8 and MMP-9 during RSV infection was the result of direct interaction of PBMCs or neutrophils with RSV,

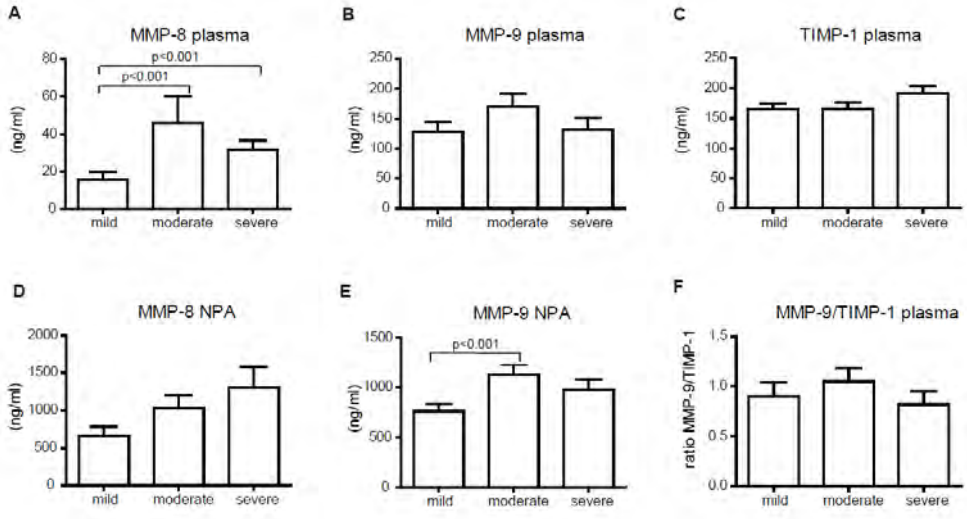

Figure 4. MMP-8 and MMP-9 concentrations in plasma and nasopharyngeal samples from children with mild, moderate, and severe RSV infection. For RSV positive children plasma concentration of MMP-8 (A), MMP-9 (B), TIMP-1 (C), and concentration in nasopharyngeal washes of MMP-8 (D) and MMP-9 (E) in mild, moderate, and severe disease are given. Ratio's between plasma MMP-9 and TIMP-1 (F). Concentrations (ng/ml) are given in mean± standard error. Mann–Whitney U-tests were performed to compare mild, moderate, and severe disease. A two-sided value of $P < 0.05$ was considered statistically significant.

PBMCs and neutrophils were stimulated with RSV in vitro. Stimulation of PBMCs with LPS (TLR4 agonist) induced MMP-9 secretion, whereas stimulation with RSV had no effect. None of the stimuli induced MMP-8 secretion by PBMC (Fig. 5A). Stimulation of PBMCs with RSV did not result in increased gene expression of MMP-8 and only a moderate increase of MMP-9 expression was observed (Fig. 5B). Unstimulated neutrophils secreted high levels of MMP-8 and MMP-9. Stimulation with LPS and RSV had no effect on the release of MMP-8 and MMP-9 by neutrophils (Fig. 5C).

DISCUSSION

This study demonstrates that disease severity of viral LRTIs in children is associated with increased gene expression levels of the MMP-8 and MMP-9 genes in both PBMCs and granulocytes. These associations were observed in children with lower respiratory tract infections caused by either RSV or other respiratory viruses. The in vitro experiments in this study show that MMP-8 and MMP-9 mRNA and protein expression in PBMCs and granulocytes is not induced by stimulation

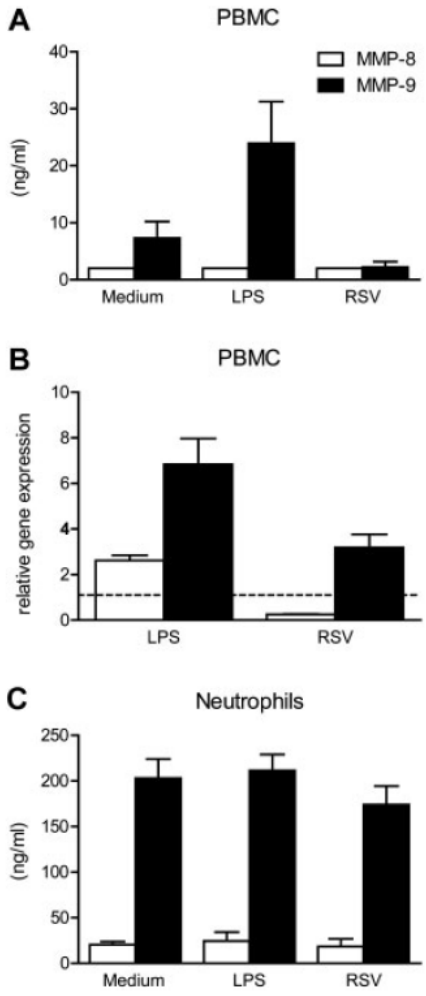

Figure 5. Stimulation of PBMCs and neutrophils by LPS and RSV in vitro. Human PBMC of healthy volunteers (n ¼ 4) were stimulated with LPS (1 ng/ml) or RSV A2 (MOI 1) and MMP-8 and MMP-9 concentrations in supernatant were measured after 24 by ELISA (A) or at transcriptional level by q-PCR (B). Neutrophils were stimulated for 4 hr and MMP levels were determined in the supernatant (C).

with RSV. Consequently, other factors than direct viral interaction induce gene expression in PBMCs and granulocytes.

This is the first study that describes an association between MMP-8 and MMP-9 gene expression and disease severity of viral LRTIs in children. This association was significant for children with a RSV infection and there was a trend for children with a viral LRTI caused by other viruses, indicating to a more general marker for disease severity during respiratory viral infections.

Several studies have shown that transcriptional analysis of peripheral blood cells can be used to discriminate the etiology and disease outcome.[29-32] Ramilo et al. compared the transcriptional profiles of PBMCs of children with infectious diseases, and identified a set of genes that could separate influenza A

infections from bacterial infections (*Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus pneumoniae*).[31] Both MMP-8 and MMP-9 were not represented in the selected set of classifier genes. Retrospective analysis of the microarray data set was performed by us, and showed that mRNA expression of MMP-8 and MMP-9 were elevated in all groups compared to controls, indicating a more general marker for inflammatory disease (data not shown). No data were available on disease severity, so it is not possible to exclude an association with disease severity. In an experimental model of viral infection of the upper respiratory tract in adults with RSV, influenza, and RV, no up-regulation of MMP-8 and MMP-9 was detected in whole blood transcriptional profiles.[33] However, these infections were all mild with consequently low levels of inflammatory markers.

This study shows that in MMP-8 plasma concentrations were increased during acute infection with RSV. Although MMP-8 plasma concentrations were higher in moderate and severe disease compared to mild disease, there was no step-wise relation with disease severity. This is in contrast with the gene expression data and indicates a different source of plasma proteins than the circulating cell population. This is in line with the study of Hartog et al. in which they found elevated MMP-8 concentrations in plasma and lung fluid in adults with hospital-acquired bacterial pneumonia compared to healthy controls.[16] They found an association between clinical severity scores and MMP-8 concentrations in bronchoalveolar lavage fluid, but not in plasma.

Although MMP-9 plasma concentrations were increased during acute viral respiratory infections in children, no association between MMP-9 plasma concentrations and disease severity was found in this study. Previous studies have described such an association for several inflammatory diseases, such as pneumonia[16], tuberculosis infections[34], septic shock[35], and asthma.[22] This may be related to the fact that, in consistence with other studies[36-38], MMP-9 concentrations were correlated to neutrophil counts, although no significant differences in neutrophil counts between the different severity groups were observed in this study.

TIMP-1 is an inhibitor of the protease activity of all known MMPs.[39] Previous studies have described an association between an imbalance between MMP-9 and TIMP-1 and tissue degradation and airflow obstruction in asthma and chronic bronchitis.[37, 40] In addition, elevated MMP-9/TIMP-1 ratios have been observed in plasma from patients with status asthmatics.[22] Furthermore, it has been shown that increased TIMP-1 concentrations, but not MMP-9, in nasopharyngeal washes of RSV-infected children correlated with disease severity and this suggests that a disturbed MMP-9/TIMP-1 homeostasis contributes to disease severity.[26] The ratio

of MMP-9 and TIMP-1 concentration in plasma did not show a correlation with disease severity in this study and indicates that MMP-9 is differentially regulated at the mucosal level during infection.

Although both MMP-8 and MMP-9 concentrations in nasopharyngeal samples were increased during acute infection compared to recovery samples no association with disease severity was observed. The wide range of nasopharyngeal concentrations between individuals is partly due to the variation induced by aspiration volumes from the nasopharyngeal cavity. Currently, more standardized methods have been developed, such as flocked swabs, which can be used for viral diagnostics as well as protein analysis.[41, 42] Normalization of protein levels to stable metabolites present in the mucus might further improve the use of nasopharyngeal samples for diagnostics, although these methods are not available yet. Further it should be taken into account that upper respiratory samples do not necessarily represent the situation in the lower airways and the systemic inflammatory response.

The in vitro experiments in this study show that MMP-8 and MMP-9 mRNA and protein expression in PBMCs and granulocytes were not induced by stimulation with RSV. Other factors than direct interaction between RSV and host cells could explain the increased gene expression levels of MMP-8 and MMP-9 in children with viral LRTIs. Influx of bone marrow-derived neutrophil precursors in blood from children with severe RSV infections can result in higher MMP-9 expression due to granule protein production, such as MMP-8 and MMP-9, during immature stages of neutrophil development.[43] Also inflammatory mediators, such as growth factors, pro-inflammatory cytokines, oxidative stress upon viral infection can induce elevated gene expression levels of MMPs.[14] It has also been shown that the lung injury caused by mechanical ventilation has resulted in increased MMP-8 and MMP-9 expression.[44] However, in this study, the last mentioned cannot completely explain the differences in gene expression since also differences in gene expression between patients with mild and moderate disease were observed, all non-ventilated patients.

The results of this study indicate that neutrophils are the major source of MMP-9 production. The higher MMP-8 and MMP-9 concentrations in nasopharyngeal samples compared to plasma may therefore reflect the influx and degranulation of neutrophils in the airways during infection.

This is in contrast to observations made by others that suggest that airway epithelial cells are the primary source of MMPs. It has been shown that MMP-9 gene expression is increased in human airway epithelial cell lines infected with RSV.[24] However, another study indicated that infected human airway epithelial

cells are not the primary source of MMPs and TIMP-1 and that infiltrating leukocytes are responsible for MMP-9 in airway samples.[26] Also in a RSV infection model in mice, of which it is known that the epithelial cells are not infected, it was demonstrated that gene expression of MMP-9 is elevated in the lungs most likely by infiltrating cells.[25] For MMP-8, no correlation with neutrophil counts was observed and gene expression levels in granulocytes and PBMCs were comparable indicating that MMP-8 transcription and secretion was different regulated than MMP-9. This is supported by differences in the degranulation of subcellular neutrophilic granules, in which MMP-8 and MMP-9 are stored and differences in transcriptional events that induce MMP-8 and MMP-9 mRNA expression.[45] For example, it has been shown that pro-inflammatory cytokines, particularly IL-1ß play a central role in the modulation of MMP-8 expression.[46, 47] Future studies may reveal the role for MMP-8 plasma concentrations as a potential biomarker to assess disease severity in viral lower respiratory tract infections in children.

The relation of MMP-8 and MMP-9 with viral load in the nasopharyngeal cavity have not been investigated in this study. In experimental respiratory viral infection models, inflammatory markers such as cytokines correlated with viral load and the symptom scores, indicating that the amount of virus is the driving force for inflammation.[48] Also in children, disease severity has been associated with high viral titers.[49, 50] Further research might reveal the role of inflammatory mediators in the pathogeneses of severe respiratory viral infections.[51] In this light, it is interesting to consider inflammatory mediators, such as MMPs, as potential targets for therapy.

Some limitations of this study need to be considered. First, the younger age of the children with the most severe lower respiratory tract infections may have caused a bias in the results. However, age was not correlated with MMP-8 and MMP-9 plasma concentrations, which suggests that age alone cannot explain the observed differences. According to these results, Thrailkill et al. did not found significant differences in MMP-8 and MMP-9 serum concentrations in children, 2–18 years of age.[52] Second, multiple viruses were detected in 40% children with lower respiratory tract infections and it cannot be entirely exclude that the presence of multiple viruses have induced a different inflammatory response.

The results of this study suggest that the expression of MMP-8 and MMP-9 genes are potential marker candidates for diagnostic use to assess disease severity in children with viral LRTIs. Markers for disease severity do not have clinical implication at present, because currently antiviral treatment is not available. However, new antiviral treatment of patients will be based most likely on diagnostics predicting disease severity and susceptibility.[53, 54] Furthermore,

markers for disease severity are also important for research purposes to study effects of interventions, such as treatment. Although currently no biomarkers at transcription level are available in the clinic, because the processing is time consuming, innovative techniques may enable rapid analysis of the expression of multiple genes at transcriptional level in the near future.[55]

In conclusion, increased expression of the MMP-8 and MMP-9 genes was observed in PBMCs and granulocytes obtained from children with severe viral lower respiratory tract infections. MMP-8 and MMP-9 gene expression levels in circulating cells may be useful markers to support clinical evaluation of disease severity in viral respiratory infections. These results justify future follow-up, that is, the validation in independent prospective study cohorts of the usefulness of MMP-8 and MMP-9 as a potential markers for disease severity in viral respiratory infections.

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Chapter 5

CD4+ T-cell counts and IL-8 and CCL-5 plasma concentrations discriminate disease severity in children with RSV infection

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ABSTRACT

Background

Current tools to predict the severity of respiratory syncytial virus (RSV) infection might be improved by including immunological parameters. We hypothesized that a combination of inflammatory markers would differentiate between severe and mild disease in RSV-infected children.

Methods

Blood and nasopharyngeal samples from 52 RSV-infected children were collected during acute infection and after recovery. Retrospectively, patients were categorized into three groups based on disease severity: mild (no supportive treatment), moderate (supplemental oxygen and/or nasogastric feeding), and severe (mechanical ventilation). Clinical data, number of flow-defined leukocyte subsets, and cytokine concentrations were compared.

Results

Children with severe RSV infection were characterized by young age; lymphocytopenia; increased interleukin (IL)-8, granulocyte colony-stimulating factor (G-CSF), and IL-6 concentrations; and decreased chemokine (C-C motif) ligand (CCL-5) concentrations in plasma. The combination of plasma levels of IL-8 and CCL-5, and CD4+ T-cell counts, with cutoff values of 67 pg/ml, 13 ng/ml, and 2.3 × 106/ml, respectively, discriminated severe from mild RSV infection with 82% sensitivity and 96% specificity.

Conclusion

This study demonstrates that the combination of CD4+ T-cell counts and IL-8 and CCL-5 plasma concentrations correlates with disease severity in RSV-infected children. In addition to clinical features, these immunological markers may be used to assess severity of RSV infection and guide clinical management.

INTRODUCTION

Respiratory syncytial virus (RSV) is a common cause of bronchiolitis in young children.[1, 2] The clinical manifestations of RSV infection range from a common cold to severe lower respiratory tract infections requiring mechanical ventilation. About 1–2% of RSV-infected children are hospitalized[3–5], of which 6–11% require intensive care admission.[3, 6] Although young age, prematurity, congenital heart diseases, chronic lung diseases, and immune deficiencies are risk factors for severe RSV infection, more than half of the RSV-infected children requiring intensive care admission were otherwise healthy.[3, 7] It has been reported that 35% of children hospitalized with bronchiolitis did not receive any supportive intervention.[8] On the other hand, it is crucial that those children who may experience clinical deterioration are not discharged. Among those sent home with a diagnosis of bronchiolitis, 4.6–6.8% required hospitalization later on during infection.[9,10] Clinical prediction models may help clinicians to distinguish RSV-infected children requiring hospitalization from those who can be safely sent home. To date, mainly clinical parameters have been used to predict the severity of disease in RSV infection.[11,12] Several studies have associated severity of RSV disease with particular cytokines such as interleukin (IL)-8, IL-6, IL-4, and interferon (IFN)-γ. [13–16] A change in plasma or nasopharyngeal levels of these cytokines in an early phase of the host response can be used as early markers for severity of disease. The addition of inflammatory parameters may improve the prediction of disease severity in children with RSV infections. To examine whether a combination of these inflammatory mediators can differentiate between severe and mild RSV infection, we characterized immune cells (CD4+ and CD8+ T cells, natural killer (NK) cells, monocytes, and B cells) and measured concentrations of 16 cytokines and chemokines in young children with RSV bronchiolitis.

METHODS

Study Design

Children below 2 years of age with RSV bronchiolitis were prospectively included during three consecutive winter seasons (from November to April 2006–2009). Bronchiolitis was defined as an acute infection of the lower airways, characterized by increased respiratory effort and expiratory wheezing and/or crackles and/or apnea. The study was approved by the Committee on Research Involving Human

Subjects of the University Nijmegen Medical Center, and written informed consent was obtained from all parents. Within 24hr after presentation, blood and nasopharyngeal samples were collected; and from hospitalized children, permission was asked of the parent or guardian for a recovery sample 4–6 wk later. Clinical data were collected from questionnaires and medical records. Retrospectively, based on the clinical course, patients were classified into three different groups: children without supportive interventions were allocated to the mild group; those requiring hospitalization for supplemental oxygen (oxygen saturations below 93%) and/or nasogastric feeding were allocated to the moderate group; and children requiring mechanical ventilation were allocated to the severe group.

Sample Collection

Nasopharyngeal aspirates were collected by introducing a catheter into the nasopharynx. Then, 1.5 ml saline was instilled into the nose and, while slowly retracting the catheter, the nasopharyngeal fluid was aspi-rated in a collection tube. Subsequently, the catheter was flushed with 1 ml saline, which was added to the collection tube. The samples were cooled and immediately transported to the laboratory. The nasopharyngeal aspirate was centrifuged and the supernatant was frozen at -80 °C. A total of 5 ml of blood was collected into sodium heparin tubes and processed within 2 h. Blood smears were stained with May-Grunwald-Giemsa to determine the percentages of granulocytes. PBMCs were obtained by density gradient centrifugation (Lymphoprep, Axis Shield, Oslo, Norway) and stored in liquid nitrogen after cryopreservation. Immunophenotyping was performed on either fresh blood cells or on viably frozen PBMCs to determine the leukocyte subsets. Plasma samples, diluted 1:1 with phosphate-buffered saline (PBS), were stored at -80 °C for cytokine analyses.

Virus Detection

Multiplex real-time reverse transcription PCR was performed on nasopharyngeal aspirates as previously described.[39] The multiplex RT-PCR assay detects 15 different viral pathogens: influenza virus type A and B, coronavirus 229E and OC43, human bocavirus, enterovirus, adenovirus, parechovirus, parainfluenza virus types 1–4, human metapneumovirus, rhinovirus, and RSV. The amount of virus was recorded semiquantitatively based on the cycle threshold (Ct) value.

Immunophenotyping

Erythrocytes were eliminated from heparinized blood by using ammonium chloride lysis; the remaining leukocytes were washed with PBS and resuspended in PBS with 0.5% bovine serum albumin (BSA). Leukocyte subsets were analyzed using immunofluorescence-labeled monoclonal antibodies (mAb) determined in multicolor flow cytometry. Depending on the number of cells, 50 µl of cell suspension containing maximally 1×10^6 nucleated cells was incubated with 50 µl fluorochrome-labeled monoclonal antibodies (final dilution 1:20) for 15 min in the dark at room temperature. Combinations of mAb conjugates were used for CD3, CD4, CD8, CD56, CD45, CD14, and CD19 (Beckman Coulter, Miami, FL). Cells were enumerated by using flow-count counting beads (Beckman Coulter). Staining of cells was determined by using a Fc500 flow cytometer (Beckman Coulter). Data were analyzed using the CXP software (Beckman Coulter). Immunophenotyping of cryopreserved PBMCs and intracellular cytokine staining were performed after thawing. The following combinations of markers and fluorescent antibodies were used: CD14–fluorescein isothiocyanate (FITC), CD16.56–phycoerythrin (PE), IL-4–PE, CD3–peridinin chlorophyll protein, CD19–allophycocyanin (APC), IFN-γ–APC, CD4–PE–Cy7, and CD8–APC–Cy7. Extracellular staining of surface markers CD14, CD16.56, CD3, CD4, and CD8 was performed in 96-well microtiter plates. For intracellular cytokine staining (IL-4 and IFN-γ), PBMCs were stimulated with β-mercapto-ethanol, phorbol myristate acetate (10 ng/ml), golgistop, and ionomycin at 37 °C for 4 h. Then, staining of surface markers CD3, CD4, and CD8 was performed. Thereafter, cells were fixed, permeabilized with 0.5% saponin and 0.5% BSA in PBS, and stained for intracellular IL-4 and IFN-γ. Samples were acquired immediately after staining on a BD FACSCanto (Becton Dickinson, Heidelberg, Germany) and analyzed using flow cytometry analysis soft-ware (FlowJo analyses 7.6, Three Star, Ashland, OR).

Cytokine Concentrations

Concentrations of the cytokines IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17, G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, and tumor necrosis factor (TNF) and the chemokines IL-8 (chemokine CXC ligand 8), IFN-γ–inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), and CCL-5 were measured by flow cytometry using the BD CBA Human Soluble Flex Set system (Becton Dickinson) according to the manufacturer's instructions. Briefly, cytokine-specific antibody–coated beads were incubated for 1 h with

25 µl plasma, supernatant of nasopharyngeal aspirates, or standard solution. Thereafter, samples were incubated with the corresponding PE-labeled detection antibodies for 2 h. After washing, samples were measured by flow cytometry. Analysis of data and quantification of cytokines were per-formed using FCAP Array software (Becton Dickinson). The detection limits were 2.3 pg/ml for IL-1β, 11.2 pg/ml for IL-2, 1.4 pg/ml for IL-4, 1.6 pg/ml for IL-6, 0.13 pg/ml for IL-10, 0.6 pg/ml for IL-12p70, 0.6 pg/ml for IL-13, 0.3 pg/ml for IL-17a, 1.6 pg/ml for G-CSF, 0.2 pg/ml for GM-CSF, 0.8 pg/ml for IFN-γ, 1.2 pg/ml for TNF, 1.2 pg/ml for IL-8, 0.002 pg/ml for CCL-5, 1.3 pg/ml for MCP-1, and 0.5 pg/ml for IP-10.

Statistics

Values are expressed as percentages for categorical variables and as mean and SE or median and interquartile range for continuous variables. For variables that were not normally distributed, the Kruskal–Wallis test was performed to compare continuous variables, followed by Mann–Whitney U-tests for individual comparisons. $\chi 2$ tests were performed to compare categorical data. A two-sided value of $P < 0.05$ was considered statistically significant. A receiver operating characteristic curve was plotted for those markers that were statistically different between severe and mild RSV infection. An optimal cutoff value for individual markers was then deter-mined with a sensitivity approaching 100% and specificity >85%. If the diagnostic marker was unable to meet the abovementioned criteria, the optimal cutoff value was adjusted so that both sensitivity and specificity approached 75%. With these optimal cutoff values, the sensitivity, specificity, and positive and negative predictive values of these markers or combination of markers were calculated. All statistical tests were performed by SPSS (Release 16; SPSS, Chicago, IL).

RESULTS

Severity of RSV Infection Is Associated With Young Age

Demographics and clinical features of the 52 included infants are presented in Table 1. Children with severe disease were significantly younger than those with mild or moderate disease (1.0 vs. 2.0 and 5.3 mo; $P < 0.01$ and $P = 0.04$, respectively). No other significant differences in clinical parameters were observed.

RSV Monoinfection Is Associated With Disease Severity, Irrespective of Viral Load
An examination of the nasopharyngeal samples detected RSV in all the samples. In 21 of 53 samples (40%), one or more other viruses than RSV were detected, of which rhino- virus was most frequently detected (n = 17). Viral coinfection occurred more often in children with mild RSV infection (73%) as compared with those with moderate (46%) and severe disease (16%; P < 0.01). No differences in RSV load were observed between the severity groups or between children with RSV monoinfections and those infected by multiple viruses.

Table 1. Demographics of children diagnosed with an RSV infection and categorized by severity of disease

	Mild (N=11)	Moderate (N=22)	Severe (N=19)	p-value
Age (mo), median (IQR)	5.3 (2.0–8.9)	2.0 (1.4–6.7)	1.0 (0.7–3.9)	0.006*
Age <3 mo	4 (36)	14 (64)	14 (74)	NS
Male (%)	7 (64)	15 (68)	14 (74)	NS
Birth weight (g), mean ± SE	3,312 ± 186	3,262 ± 157	3,167 ± 191	NS
Prematurity ≤35 wk	1 (9)	2 (9)	6 (32)	NS
Breastfeeding	8 (73)	12 (63)	8 (44)	NS
Smoking during pregnancy	1 (9)	3 (15)	5 (28)	NS
Congenital heart disease	0	1 (5)	1 (5.3)	NS
Atopic disease	2 (18)	2 (9)	2 (11)	NS
Siblings	7 (64)	13 (59)	17 (90)	NS
Day care	3 (38)	3 (14)	1 (5)	NS
Passive smoking	2 (20)	2 (10)	3 (18)	NS
Family history of atopy	7 (64)	13 (65)	12 (67)	NS
Onset of symptoms in days, median (IQR)	6 (4–8)	4 (3–5.25)	5 (3–6)	NS
Ct value RSV, mean ± SE	29.7 ± 1.6	28.5 ± 0.9	28.7 ± 0.9	NS
Coinfection	8 (73)	10 (46)	3 (16)	0.007**

Data are presented as number (%), unless otherwise specified. Kruskal–Wallis tests were performed and results are presented; P < 0.05 was considered to be statistically significant. If differences were significant, a Mann–Whitney U-test was performed for one-to-one comparisons: *p=0.04: mild vs. moderate; p=0.003: mild vs. severe; and **p=0.002: mild vs. severe. Ct, cycle threshold; IQR,interquartile range; NS, not significant; RSV, respiratory syncytial virus.

Severity of RSV Infection Is Associated With Lymphocytopenia

No differences were found in the numbers of granulocytes, monocytes, or B cells between the severity groups (Figure 1). Severe RSV infection was associated with lower CD4+ T-cell, CD8+ T-cell, and NK-cell counts as compared with mild or

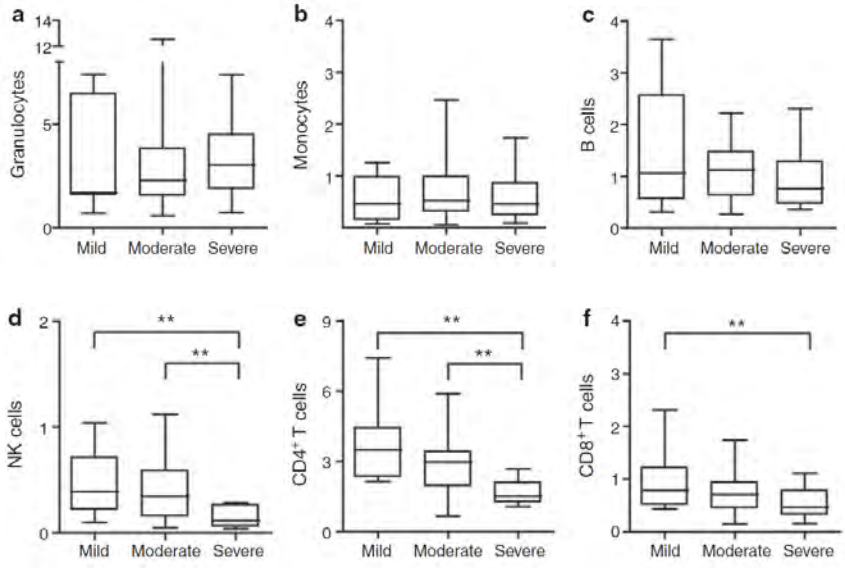


Figure 1. Absolute cell counts of leukocyte subsets in blood from children with an RSV infection categorized by disease severity. Cell counts of (a) granulocytes, (b) monocytes, (c) B cells, (d) NK cells, (e) CD4+ T cells, and (f) CD8+ T cells are expressed as 106 cells/ml. Data are plotted as medians with the 25th–75th percentile range. Mann–Whitney U-tests were performed, **p<0.01. NK, natural killer; RSV, respiratory syncytial virus.

moderate infection. During recovery, the number of NK cells, CD4+ T-cells, and CD8+ T-cells in children with severe RSV infection increased to normal values for age (Supplementary Figure S1). No differences were found in immunophenotyping of fresh or cryopreserved PBMCs (data not shown) .

Severity of RSV Infection Is Associated With High IL-8, IL-6, and G-CSF, and Low CCL-5 Plasma Concentrations

Cytokine concentrations in plasma and nasopharyngeal aspirates in the three patient groups are summarized in Table 2 and Supplementary Table S1. Cytokines that were present in less than 30% of the acute blood and nasopharyngeal samples were excluded from further analyses (Supplementary Tables S2 and S3). In children with severe disease, IL-8, IL-6, and G-CSF plasma concentrations were higher than in those with mild or moderate disease (Table 2). In contrast, severe disease was associated with lower CCL-5 plasma concentrations as compared with mild or moderate disease ($p<0.01$). Whereas CCL-5 concentrations were higher during acute infection as compared with those during recovery in the

moderate group (p=0.03), CCL-5 plasma levels were lower during acute infection as compared with those during recovery in the severe group (p=0.01).
In addition, CCL-5 plasma concentrations during recovery were significantly higher in the moderate group than in the severe group (p=0.03). No significant differences in the percentage or absolute numbers of IL-4– and IFN-γ–producing CD4+ and CD8+ T cells were observed in children with severe RSV infection as compared with those with mild or moderate RSV infection (Supplementary Table S4).

Table 2. Cytokine and chemokine concentrations (pg/ml) in plasma of children with mild, moderate, or severe RSV infection

	Mild (N=11)	Moderate (N=20)	Severe (N=19)	p-value
IL-8	48.1 (36.0–76.8)	55.7 (40.5–106.6)	127.8 (79.0–321.9)	<0.0001*
CCL-5	25507 (19971–35204)	25235 (11264–45161)	8632 (3895–12155)	0.007**
IL-6	20.4 (11.0–35.6)	10.1 (0.0–69.8)	72.9 (21.9–122.4)	0.025***
IL-1	21.7 (0.0–37.0)	6.0 (0.0–38.9)	0.0 (0.0–23.6)	NS
MCP-1	220.0 (168.1–296.4)	138.4 (106.7–229.9)	174.6 (103.9–481.0)	NS
G-CS	51.9 (21.2–180.8)	47.0 (12.9–111.1)	129.9 (85.6–362.7)	0.021†
IP-10	575.6 (418.9–902.8)	395.7 (331.9–666.5)	660.4 (347.3–1,184.9)	NS

Data are presented as median values (25th–75th percentile). Kruskal–Wallis tests were performed and the results are presented; p<0.05 was considered to be statistically significant. If differences were significant, a Mann–Whitney U-test was performed for one-to-one comparisons: *p<0.01: mild vs. severe and moderate vs. severe; **p<0.01: mild vs. severe and moderate vs. severe; ***p=0.02: moderate vs. severe; and †p=0.01: moderate vs. severe. CCL, chemokine (C-C motif) ligand; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; IP, inducible protein; MCP, monocyte chemoattractant protein; NS, not significant; RSV, respiratory syncytial virus.

Severity of RSV Infection Is Associated With High IL-6 Nasopharyngeal Concentrations

Overall, nasopharyngeal cytokine concentrations were higher during acute infection than after recovery (Supplementary Table S3 online). Higher IL-6 concentrations were found in nasopharyngeal samples from children with moderate or severe disease as compared with those with mild disease ($P < 0.05$). No other significant differences in nasopharyngeal cytokine levels were found between the patient groups (Supplementary Table S1).

Inflammatory Markers in RSV Infection Inflammatory Parameters Distinguish Disease Severity in RSV-Infected Infants Younger Than 3 months and in Children With Single RSV Infection

Given that severe disease was associated with younger age, we also performed analyses in infants younger than 3 months. Because of the small numbers, we categorized these infants into two groups: ventilated (N=15) and nonventilated (N=18). In this young age group, higher IL-8, IL-6, and G-CSF plasma concentrations, lower CCL-5 plasma concentrations, and lower NK cell and CD4 T-cell counts were observed in ventilated as compared with nonventilated RSV-infected infants (Table 3).

Table 3. Significant differences in clinical and inflammatory parameters between ventilated and nonventilated RSV-infected infants younger than 3 months

	Ventilated (N=15)	Nonventilated (N=18)	p-value
Age (days)	31.0 (19.0–48.7)	53.4 (45.0–59.7)	0.01
Plasma IL-8	127.8 (87.4–137.8)	57.3 (40.2–43.9)	<0.01
Plasma IL-6	66.4 (3.2–98.0)	12.9 (3.2–43.8)	0.03
Plasma G-CSF	114.5 (3.2–157.3)	34.3 (6.1–144.6)	0.03
Plasma CCL-5	8,874.2 (8,193.6–38,503.2)	12,529.0 (5,863.3–15,769.5)	0.01
Nasopharyngeal IL-6	291.7 (152.0–522.1)	335.1 (229.7–368.8)	0.03
NK cells	0.12 (0.06–0.27)	0.41 (0.20–0.58)	<0.01
CD4+ T cells	1.67 (1.24–2.25)	2.80 (2.32–3.56)	<0.01
CD4/CD8 ratio	2.60 (2.60–3.78)	5.28 (3.31–7.91)	0.01

Numbers are presented as median values (25th–75th percentile). Cytokine concentrations are given in pg/ml and cell numbers in 10^6 cells/ml. Mann–Whitney U-tests were performed; $p<0.05$ was considered to be statistically significant. CCL, chemokine (C-C motif) ligand; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; NK, natural killer; RSV, respiratory syncytial virus.

As infection by multiple viruses may have influenced our results, we analyzed inflammatory parameters in 16 ventilated and 15 nonventilated children with RSV single infections. No significant differences in age and clinical parameters were found. Significantly higher IL-8, IL-6, and G-CSF and, although not significant, lower CCL-5 plasma concentrations ($p=0.06$) were observed in ventilated children as compared with nonventilated children. In addition, CD4+ T-cell and NK cell but not CD8+ T-cell counts were lower in ventilated children than in nonventilated children.

Combination of IL-8, CCL-5, and CD4+ T-cell Count Discriminates Severe RSV Infection From Mild RSV Infection

Clinical and inflammatory parameters that were significantly different between the patient groups (Table 4) were selected for further analyses. On the basis of

Table 4. Comparison of sensitivity, specificity, PPVs, NPVs, and AUC for different inflammatory markers in ventilated versus nonventilated children with RSV bronchiolitis

Marker	Cutoff value	Sensitivity	Specificity	PPV (%)	NPV (%)	AUC
CCL-5	≤13 ng/ml	0.79	0.74	65	85	0.782
IL-8	≥67.2 pg/ml	0.89	0.77	68	92	0.844
IL-6	≥28.7 pg/ml	0.75	0.67	60	80	0.718
G-CSF	≥82.7 pg/ml	0.80	0.67	62	83	0.723
Age	≤2 mo	0.65	0.57	48	73	0.718
CD4+ cells	≤2.29 × 106/ml	0.87	0.74	87	74	0.859
NK cells	≤0.28 × 106/ml	0.87	0.63	87	63	0.812
Performance of combined markers						
IL-8 and CD4		0.80	0.89	80	89	
CCL-5 and CD4		0.91	0.84	67	96	
≥2 of IL-8, CCL-5,and CD4a		0.82	0.96	93	89	

Cutoff values are based on optimal sensitivity and specificity for each marker. AUC, area under the curve; CCL, chemokine (C-C motif) ligand; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; NK, natural killer; NPV, negative predictive value; PPV, positive predictive value; RSV, respiratory syncytial virus. The combination of IL-8, CC L-5, and CD4+ T-cell counts discriminated ventilated from nonventilated children under the condition that two or more cutoff values of IL-8 and CC L-5 concentrations and CD4+ T-cell counts were exceeded.

the receiver operating characteristic (ROC) curves, cutoff values were calculated (67 pg/ml, 13 ng/ml, and 2.3 × 106 cells/ml for IL-8 and CCL-5 plasma levels and CD4 T-cell counts, respectively).

IL-8 plasma levels and CD4+ T-cell counts showed high sensitivity (89% and 87%, respectively) and specificity (77% and 74%, respectively), followed by CCL-5 with a sensitivity of 79% and specificity of 74%. We explored the possibility of using a combination of markers to predict disease severity. The combination of IL-8 and CCL-5 levels and CD4+ T-cell counts predicted disease severity with a sensitivity of 82% and specificity of 96%, if two of three cutoff values within a patient were above or below the threshold. Positive and negative predictive values were 93% and 89%, respectively, for this combination of markers.

DISCUSSION

This study demonstrates that CD4+ T-cell counts and IL-8 and CCL-5 plasma concentrations may be a useful set of markers for distinguishing disease severity in young children with RSV bronchiolitis. Therefore, by including immunological inflammatory parameters, current clinical prediction models for severity of RSV

infection may be improved.

In our study, young age, lymphocytopenia, and IL-8, IL-6, G-CSF, and CCL-5 plasma levels were associated with severe RSV infection. Young age is a known risk factor for severe RSV infection. An immature immune system, in combination with the lack of in utero sensitization to RSV and small airways, indicates that severe RSV infections are predominantly observed in young infants.[17] However, age alone cannot explain the observed differences in inflammatory parameters because our analyses in infants younger than 3 months still showed significant differences in inflammatory parameters between the severity groups.

In line with previous studies, lower T-cell and NK-cell counts were observed during severe RSV infection as compared with milder infections.[18-20] Given that young infants have normally higher T-cell and NK-cell counts than older children, the degree of lymphocytopenia may even have been underestimated in this patient group.[21,22] This lymphocytopenia can be explained by either migration of lymphocytes into the airways or apoptosis.[13,18,19,23] Another hypothesis may be that infection-induced immune suppression results in lower lymphocyte counts.

As previously described[15], our results show that RSV infections induce a systemic response reflected by changes in cytokine plasma concentrations. Despite the significantly higher IL-8, IL-6, and G-CSF and lower CCL-5 concentrations in plasma during severe RSV infection as compared with milder infection, in nasopharyngeal aspirates, only IL-6 was significantly higher during severe RSV infection. Given that nasopharyngeal samples do not necessarily reflect local inflammation in the lower airways, and sampling methods and correction for dilutional effects differ between studies, the use of cytokines in nasopharyngeal samples as biomarkers is complicated.[24] Because blood samples are relatively easy to obtain in a uniformly established manner, and cytokine plasma levels were all normalized at the time of recovery, cytokine levels in plasma are potential biomarker candidates for diagnostic use.

In contrast to increased IL-8, IL-6, and G-CSF levels, CCL-5 plasma concentrations were decreased during severe RSV infection as compared with those after recovery and milder manifestations of RSV infection. Although our results differ from some studies that observed increased CCL-5 concentrations in respiratory samples[24, 25] and blood[26] during acute RSV infection as compared with healthy controls, they are consistent with others showing lower CCL-5 concentrations in blood during acute infection as compared with recovery samples.[27,28] The fact that not all studies examined recovery samples or evaluated disease severity may explain these differences.

Although CCL-5 is initially produced by local innate immune cells to attract T

cells and monocytes to the site of infection, CD4+ and CD8+ T cells have been described as the main source of the second peak of CCL-5, which occurs 5–7 d after infection.[29] A decrease in T cells may therefore result in lower CCL-5 levels. However, the discrepancy between T-cell counts and CCL-5 levels during recovery in the moderate and severe groups suggests that the lower T-cell counts cannot completely explain the lower CCL-5 levels during severe RSV infection. Other mechanisms such as an inadequate or suppressed T-cell response may play a role. Infection-induced immune suppression is a well-established phenomenon and has been described in sepsis, measles, and chronic viral infections.[30-32] The immature immune system of infants can also result in a reduced immune response. Controversial results have been published on changes in CCL-5 levels during the first months of life, and we cannot exclude that higher CCL-5 levels reflect functional maturation of the immune system.[33, 34] However, in infants younger than 3 months, we still observed significantly lower CCL-5 concentrations in ventilated infants than in non-ventilated infants.

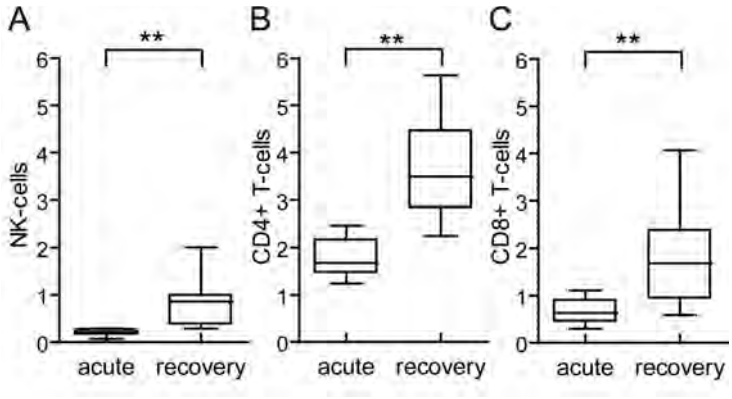
The inflammatory response during severe RSV infection has been associated with atopy and asthma, although the precise mechanism behind this relation is still unclear.[35] It has been suggested that an IL-4–induced Th2 response during primary RSV infection might play a role in the development of atopic disease, although this could also be due to a shared etiology of bronchiolitis and asthma.[36] If this had played a role in our study, one would expect differences in IL-4–producing T-cell subsets or differences in the prevalence of atopic disease. However, no differences between severe and nonsevere patients were found. Therefore, it is unlikely that the inflammatory markers found to be associated with disease severity can be explained by the existing atopy or the development of atopic disease.

Some limitations of our study need to be considered. Multiple viruses were detected in 40% of the samples, and these co-infections were associated with less severe RSV infection. Although comparing single RSV infections between ventilated and non-ventilated children gave results similar to those of the analyses in which multiple infections were included, the possibility that the presence of other viruses induced a different inflammatory response cannot be entirely excluded. Another important consideration regarding markers for disease severity is that they do not have clinical implications at this moment, because currently no antiviral treatment is available. However, newly developed antiviral treatment of patients will be based most likely on diagnostics predicting disease severity and susceptibility.[37,38] For the evaluation of intervention studies, such as vaccination and antiviral treatment, markers for disease severity will be valuable.

Furthermore, given that we focused on the discrimination between ventilated and nonventilated children, studies need to be undertaken to determine whether this set of biomarkers could also discriminate between children requir-ing hospitalization and those who can be discharged.

In summary, we demonstrate the usefulness of a combination of immunological markers to distinguish disease severity in young RSV-infected children. We conclude that prediction models of RSV infection in young children may be improved by including immunological parameters, such as IL-8 and CCL-5 concentrations and CD4+ T-cell counts. Future studies have to be performed to validate our findings to see whether the use of these parameters may contribute to early recognition of children developing severe RSV infection. This may guide clinicians to decide whether a child needs to be hospitalized and to initiate symptomatic therapy in an early phase of disease.

SUPPLEMENTARY DATA



Supplementary Figure S1. Absolute cell counts during acute infection and after recovery in mechanically ventilated children. Cell counts of NK-cells (A), CD4+ T-cells (B) and CD8+ T-cells (C) are expressed as 106 cells/ml. Data are plotted s medians with 25th-75th percentile range. Paired analyses using Wilcoxon signed-rank test were performed, ** p<0.01.

Supplemental Table S1. Cytokine and chemokine concentrations (pg/ml) in nasopharyngeal aspirates of children with mild, moderate and severe RSV infection.

	mild (N=9)	Moderate (N=13-15)	Severe (N=14-15)	p-value
IL-8	9106 (7599-18947)	11908 (10672-20307)	17773 (11917-67609)	NS
CCL-5	116.8 (52.6-179.9)	74.0 (57.5-208.5)	48.0 (0.0-199.8)	NS
IL-1β	552.4 (206.5-944.4)	1670.4 (763.5-3150.9)	1093.7 (545.3-5932.5)	NS
IL-6	1475.20 (1136.6-1908.4)	2593.3 (1641.6-4492.8)	4801.2 (1520.3-8186.6)	p=0.031*
IL-10	28.9 (0.0-49.6)	101.7 (0.0-207.8)	22.7 (0.0-139.4)	NS
MCP-1	140.7 (60.4-251.2)	394.1 (202.6-394.1)	539.9 (89.2-1865.7)	NS
TNF	216.1 (168.4-356.0)	649.3 (266.2-1990.1)	431.2 (251.1-2290.9)	NS
G-CSF	4940.4 (3416.0-8715.8)	5001.6 (3220.8-8403.7)	3242.7 (1437.1-5780.9)	NS
IP-10	11788 (7168-24024)	14633.9 (5289.1-24037.4)	5016.0 (775.9-11538.9	NS

Data are presented as median (25th-75th percentile). Kruskal-Wallis tests were performed and presented in the table, p<0.05 was considered to be statistically significant. If differences were significant a Mann Whitney U test was performed for one to one comparisons: * p<0.05: Mild vs. severe and moderate vs. severe

Supplemental Table S2. Paired analyses of cytokine and chemokine plasma concentrations (pg/ml) during acute RSV infection and after recovery in moderate and severe disease groups.

	moderate acute	**moderate recovery**	**N**	**p-value**
IL-8	60.7 (49.7-126.2)	9.9 (6.3-14.0)	12	<0.01
CCL-5	20312 (11846-45161)	12872 (9583-32677)	12	0.03
IL-6	22.6 (2.7-76.9)	0.0 (0.0-0.0)	11	0.02
IL-10	30.7 (0.0-54.7)	0.0 (0.0-0.0)	12	0.01
MCP-1	159.2 (106.7-296.1)	146.4 (87.0-198.8)	12	0.05
G-CSF	47.0 (13.1-136.0)	5.8 (0.0-14.5)	11	0.05
IP10	413.5 (365.2-794.0)	191.5 (128.8-246.2)	11	<0.01
	severe acute	**severe recovery**	**N**	**p-value**
IL-8	130.7 (90.7-326.5)	12.2 (1.4-25.0)	16	<0.001
CCL-5	8874 (3314-20488)	34351 (21253-61944)	15	<0.01
IL-6	79.4 (9.7-146.1)	0.0 (0.0-0.0)	15	<0.01
IL-10	7.4 (0.0-23.5)	0.0 (0.0-0.0)	16	0.01
MCP-1	176.1 (107.1-482.4)	232.4 (148.6-358.4)	16	NS
G-CSF	129.9 (85.6-371.7)	3.9 (0.0-10.5)	15	<0.01
IP10	660.4 (437.3-1286.7)	187.6 (133.8-292.3)	15	<0.01

Data are presented as median (25th-75th percentile). Wilcoxon signs ranks test were performed for paired analyses between acute and recovery samples, $p<0.05$ was considered to be statistically significant.N=number of subjects, NS= not significant.

Supplemental Table S3. Paired analyses of cytokine and chemokine nasopharyngeal concentrations (pg/ml) during acute RSV infection and after recovery in moderate and severe disease groups.

	moderate acute	**moderate recovery**	**N**	**p-value**
IL-8	14971 (11314-24860)	8758.2 (1449.8-15201.0)	7	0.03
CCL-5	74.0 (40.7-176.5)	23.3 (0.0-60.1)	5	0.04
IL-1B	1743.0 (763.5-3236.5)	68.4 (24.0-868.6)	5	0.04
IL-6	2001.6 (1616.3-3518.4)	215.9 (64.4-559.7)	7	0.02
IL-10	103.8 (21.1-412.8)	0.0 (0.0-51.2)	5	NS
MCP-1	302.8 (159.0-373.9)	32.3 (7.0-65.7)	4	NS
TNF-a	823.5 (200.4-1944.9)	2.4 (0.0-70.7)	4	NS
G-CSF	6090.0 (3623.1-9296.7)	3590.1 (449.1-4922.1)	7	NS
IP-10	18229 (8009-28110)	3321.0 (1897.8-9194.0)	7	0.02
	severe acute	**severe recovery**	**N**	**p-value**
IL-8	16072 (10196-41044)	7528.3 (2973.5-14524.0)	10	0.05
CCL-5	48.0 (3.0-435)	20.8 (0.0-107.5)	9	NS
IL-1B	1033.0 (604.5-9572.0)	204.4 (144.5-880.0)	8	0.05
IL-6	4069.3 (1159.2-9814.7)	363.5 (78.4-657.3)	10	0.01
IL-10	7.7 (0.0-355)	0.0 (0.0-80.2)	5	NS
MCP-1	781.0 (68.8-3412.2)	61.3 (0.0-162.3)	7	0.02
TNF-a	431.2 (288.5-5475.3)	144.0 (0.0-378.6)	8	NS
G-CSF	3246.1 (1431.9-7609.3)	3038.1 (1327.2-5827.4)	10	NS
IP-10	3930.5 (424.5-18245.6)	1326.4 (489.1-2806.1)	10	NS

Data are presented as median (25th-75th percentile). Wilcoxon signs ranks test were performed for paired analyses between acute and recovery samples, $p<0.05$ was considered statistically significant.N=number of subjects, NS= not significant.

Supplemental Table S4. IL-4 and IFN-γ producing CD4 and CD8 positive T-lymphocytes from children with severe and non-severe RSV infection measured by intracellular cytokine staining.

	CD4+ T-lymphocytes			**CD8+ T-lymphocytes**		
	IFN-γ (%)	IL-4 (%)	IFN-γ/IL-4	IFN-γ (%)	IL-4 (%)	IFN-γ/IL-4
Non severe (N=9)	0.17 (0.07-0.26)	0.29 (0.26-0.47)	0.43 (0.30-0.69)	2.17 (1.15-2.94)	0.48 (0.29-0.56)	3.15 (2.74-6.75)
Severe (N=10)	0.11 (0.06-0.18)	0.33 (0.22-0.51)	0.39 (0.14-0.56)	2.14 (1.04-3.24)	0.59 (0.52-0.63)	2.73 (1.85-5.46)
P-value	NS	NS	NS	NS	NS	NS

Cell numbers are given in 10^6/ml, data are presented as median (25th-75th percentile). Mann-Whitney U tests were performed, $p<0.05$ was considered to be statistically significant.NS= not significant.

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TRANSCRIPTOMICS AND PROTEOMICS

Chapter 6

Olfactomedin-4 serves as a prognostic marker for disease severity in pediatric respiratory syncytial virus infection

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ABSTRACT

Background

Respiratory viral infections follow an unpredictable clinical course in young children ranging from a common cold to respiratory failure. The transition from mild to severe disease occurs rapidly and is difficult to predict. The pathophysiology underlying disease severity has remained elusive. There is an urgent need to better understand the immune response in this disease to come up with biomarkers that may aid clinical decision making.

Methods

In a prospective study, flow cytometric and genome-wide gene expression analyses were performed on blood samples of 26 children with a diagnosis of severe, moderate or mild respiratory syncytial virus (RSV) infection. Differentially expressed genes were validated using quantitative PCR (qPCR) in a second cohort of 80 children during three consecutive winter seasons. FACS analyses were also performed in the second cohort and on recovery samples of severe cases in the first cohort.

Results

Severe RSV infection was associated with a transient but marked decrease in CD4+ T, CD8+ T, and NK cells in peripheral blood. Gene expression analyses in both cohorts identified Olfactomedin4 (OLFM4) as a fully discriminative marker between children with mild and severe RSV infection, giving a PAM cross-validation error of 0%. Patients with an OLFM4 gene expression level above -7.5 were 6 times more likely to develop severe disease, after correction for age at hospitalization and gestational age.

Conclusion

By combining genome-wide expression profiling of blood cell subsets with clinically well-annotated samples, OLFM4 was identified as a biomarker for severity of pediatric RSV infection.

INTRODUCTION

Respiratory viral infections are an important cause of hospitalization among children younger than 5 years of age. Human respiratory syncytial virus (RSV) is the most common (40-85%) identified virus in infants hospitalized for respiratory infections during winter epidemics, with hospitalization rates between 1 and 2%.[1-6] Clinical manifestations range from common colds to severe lower respiratory tract infections (LRTIs) requiring mechanical ventilation. Risk factors for a severe course are known, but the majority of patients admitted to an intensive care unit were previously healthy.[7-9] Since transition from mild to severe disease can occur within hours, one of the key challenges for clinicians is to differentiate children who need hospitalization for supportive care from those who can safely be discharged. Currently, young infants with mild bronchiolitis, especially those younger than 3 months of age, are often admitted to a hospital since they have an increased risk of severe disease. However, up to 35% of children hospitalized with bronchiolitis do not receive any supportive intervention.[10] Conversely, it is crucial to avoid early discharge of those children who may experience clinical deterioration. Among children sent home with the diagnosis bronchiolitis, 4.6-6.8% require hospitalization later on.[11, 12]

Much research has been done on the immune response against RSV in humans. Several reports suggested an important role for the innate immune system, while others found an inadequate adaptive immune response especially in young children and in individuals who present with a severe clinical picture.[13] The uncertainty in the nature of the immune response against RSV is reflected in the unpredictable clinical course of the infection as well as in the difficulty of developing an adequate vaccine. We, as well as others, previously reported that T-lymphocytes can be markedly decreased in the more severe cases of the disease. We reported that in severe cases both CD4 and CD8 T-cell numbers, as well as NK-cells were reduced in peripheral blood.[14] However, it remains unclear whether this indicates an inadequate immune response against RSV, for instance by massive apoptosis or decreased production of T-cells, or that peripheral blood poorly reflects an ongoing immune response that might be very active.

The detection and application of biomarkers to assess severity of viral LRTIs, in particular RSV infection, may assist clinicians in the prediction of severe disease in children with bronchiolitis and may help to reduce the number of unnecessary hospitalizations or clinical deterioration after discharge. Furthermore, markers for disease severity are important research tools to study effects of interventions by new therapies or to stratify patients by disease severity.[15, 16] Several studies

have shown that transcriptional analysis of peripheral blood cells may be used to define different etiologies of disease and disease outcomes.[17-20] In a seminal proof-of-concept study, Ramilo et al. (2007) compared the transcriptional profiles of PBMCs of children with infectious diseases and identified a set of genes that can separate influenza A infections from bacterial infections (*Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pneumoniae)*.[20] Recently this group also reported that these transcriptome profiles also contained information regarding viral etiology (influenza, rhinovirus and RSV) and the course of disease.[21]
This study was initiated to obtain insight into the changes occurring in adaptive and innate immune cells during RSV infection and to identify possible biomarkers of disease severity. To identify transcriptional biomarkers to separate mild from severe disease, genome-wide gene expression analyses were performed on blood samples of 26 children with a diagnosis of severe, moderate or mild RSV infection in two winter seasons. A validation cohort of 80 children spanning three other consecutive winter seasons by flow cytometry and q-PCR was used to validate various candidate biomarkers.

METHODS

Study Design

In this prospective cohort study, 3 ml of sodiumheperanized blood and nasopharyngeal samples were obtained from two cohorts of patients with viral bronchiolitis within 24 hours after first contact with the hospital. Medical history, demographic data, and clinical assessments were collected from questionnaires and medical records. Presence of 15 different viral pathogens was tested by multiplex RT-PCR on nasopharyngeal samples as previously described.[22] Patients were classified retrospectively into three groups based on severity of disease. The mild group included children without hypoxia or severe feeding problems. The moderate group included children requiring hospitalization for supplemental oxygen (oxygen saturations <93%) and/or nasogastric feeding. Children requiring mechanical ventilation were included in the severe group. Recovery samples were obtained 4-6 weeks after acute infection from children with moderate and severe disease. The first cohort consisted of 31 patients with RSV infections, divided into mild (n=9), moderate (n=11) and severe (n=11) disease. This cohort was used for micro-array analysis and initial qPCR validation of genes of interest. The second cohort comprised 80 children with viral LRTIs also divided into three

groups: mild (n=14), moderate (n=42) and severe (n=24). This cohort was meant for validation purposes. All subjects were recruited at two hospitals in Nijmegen, the Canisius Wilhelmina Hospital and the Radboud University Medical Center, The Netherlands. The study protocols were approved by the Regional Committee on Research involving Human Subjects Arnhem-Nijmegen (serving as the IRB) and were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from the parents of all children.

RNA Isolation and Microarray Gene Expression Analyses

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep®, Axis Shield, Norway), counted and subsequently stored in Trizol® reagent (Invitrogen, The Netherlands) at -80˚C in the same laboratory by the same team for both cohorts. RNA from PBMC and granulocyte fractions was extracted using Trizol (Invitrogen Life Technologies) according to the manufacturer's protocol. Total RNA was isolated using the RNeasy Minikit (Qiagen). RNA integrity and quality was assessed using capillary electrophoresis [RNA 6000 Nano LabChip (Agilent)] on an Agilent Bioanalyzer 2100 system. RNA processing, target labeling and hybridization to gene expression arrays was performed by standard methods as described. [23] Biotin labeled cRNA was obtained using the One-Cycle Eukaryotic Target Labeling Assay (Affymetrix), after which 15 µg of fragmented, biotin labeled cRNA was hybridized to Affymetrix® GeneChip® Human Genome U133 plus 2.0 arrays according to standard Affymetrix protocol (Affymetrix Inc, Santa Clara, CA).

Flow Cytometry

Whole blood was stained with monoclonal antibodies (mAbs) to identify the following subsets: CD4+ T-cells (CD4+CD3+CD8-), CD8 T-cells (CD8+CD4-CD3+), NK-cells (CD3-CD56+), B-cells (CD45+CD19+), monocytes (CD14+) and granulocytes (CD14-CD45+CD15+). Samples were acquired immediately after staining on a BD FACS CANTO and analyzed using FlowJo software.

Data Analysis

Quality control analyses were performed as previously described.[23, 24] Scanned images were inspected for artifacts, percentage of calls present (<25%) and controls of RNA degradation. This led to some arrays being discarded. On each

remaining array, probes labelled outliers by the Affymetrix scanning software and overexposed probes (with maximum PM intensity level >63.000) were removed. Subsequently, probesets with less than 8 probes remaining were discarded. For each comparison, robust multichip analysis (RMA) was used for background removal, quantile normalization of probe intensity levels and probe set summarization. The resulting values were log2-transformed for further analysis, giving probeset expression levels between 0 and 16. We then selected only those probesets that showed at least a two-fold difference (up or down) on a minimum of two arrays with respect to the median expression over all arrays in that particular comparison.[24, 25] Finally, Significance Analysis of Microarrays (SAM)[26] was applied to find differentially expressed probesets with a significance level of q <0.05. To select only biologically relevant changes, we demanded additionally that the difference in absolute expression levels between groups was larger than log2(200) and that the absolute difference was >2 fold.
In a subsequent supervised analysis, we trained a PAM classifier ("Prediction Analysis of Microarrays")[27], attempting to find a minimum number of discriminative genes that yielded an optimal cross-validation error (i.e. the predicted test error). For visualization purposes, samples were clustered based on selected probesets by complete linkage hierarchical clustering with 1-correlation as a distance measure, using the Matlab Bioinformatics toolbox (Mathworks, Natick, MA). The original and processed data were deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo). All microarray experiments were performed according to the MIAME guidelines.

RT-PCR

Real-time quantitative PCR was used to measure the expression of genes of interest. Initial validation of gene expression of OLFM4 detected in the first cohort was performed with SYBR Green PCR Mastermix (Applied Biosystems; P/N 4367659) with forward 5`- atcaaaacacccctgtcgtc- 3`and reverse 5`-gctgatgttcaccacaccac-3` primers for OLFM4. Actin was used as a reference gene with forward primer 5`- cgtcacacttcatgatggagttg-3` and reverse primer 5`-cttccttcctgggcatgga-3`. After validation of the microarray, the second cohort was analyzed with commercially available Taqman primers (OLFM4 Hs00360669_m1 and GAPDH Hs99999905_m1). All samples were run for 40 cycles in duplicate. Ct values of OLFM4 were normalized against the reference gene GAPDH.

OLFM4 Plasma Measurement

OLFM4 concentrations were measured in randomly selected plasma samples of 49 patients from the validation cohort by a commercial ELISA kit (E90162Hu, Uscn Live Science Inc., China) according to the instructions of the manufacturer.

Published Microarray Data Mining

A data mining search was performed in NCBI GEO and in EBI Arrayexpress, online databases with datasets and profiles of previously performed microarray studies to validate our results.[28, 29] Terms for searching were: OLFM4, Affymetrix, whole blood children, RSV and/or homo sapiens. More than 90 microarray studies were found. Based on the population (children/infants), sample size, disease type and available information per sample, 18 studies were selected. From the series matrix files, the results were log transformed and OLFM4 gene expression was selected and analyzed to gain insight in its behavior in different disease states and ages.

Statistics

The distributions of categorical variables are presented as percentages per category. Numerical variables are reported as means with standard deviation (SD) or medians with interquartile ranges (IQR) depending on whether or not the variables were normally distributed (Kolmogorov-Smirnov's test, $p>0.05$). To determine whether OLMF4 was independently associated with receiving mechanical ventilation, multivariable log-binomial regression analyses were performed in the validation cohort resulting in adjusted Relative Risks (RR).[30]

RESULTS

We previously reported that RSV infection, especially in severe cases, was associated with lymphopenia. This was not only visible in NK- and CD8+ T-cells, known to be directly involved in anti-viral immunity, but surprisingly also in CD4+ T-cells, whereas B-cells were unaffected.[14] In the current study, we analyzed recovery samples of 6 severe patients after clearance of the infection (on average 4 weeks after discharge) and found that the numbers of NK cells as well as CD4+ and CD8+ T-cells had returned to normal, indicating that the lymphopenia was transient (data not shown).

Table 1. Patient characteristics

	Mild (N=9)	Moderate (N=9)	Severe (N=8)	p-value
Age (months)	8.7 [3.6-9.3]	1.9 [1.5-8.3]	2.4 [1.1-4.9]	NS
Gender (male)	6 (67%)	8 (89%)	6 (75%)	NS
Gestational age (wks)	40 [36.9-41.0]	38.6 [37.2-40.0]	35.1 [33.1-39.8]	NS
Length of stay (days)	0 [0-3]	5 [2-9]	13 [6.3-19.8]	p<0.001*

Values are given in numbers (percentages) and median with inter quartile range (IQR). P-values are based on Kruskall Wallis tests, followed by Mann Whitney U tests for individual comparisons: *mild vs moderate p<0.01, moderate vs severe p<0.05, mild vs severe p<0.001

Initially, genome-wide expression analyses were done on the 31 patients of the first cohort. Two and three patients from the moderate and severe groups, respectively were excluded due to insufficient RNA quality and labelling. Therefore, clinical, laboratory and microarray data of 9 patients with mild disease, 9 with moderate disease and 8 with severe disease were analysed. Patients with a clinical diagnosis of mild disease were older at time of admission to hospital than those with severe disease. The length of stay in hospital increased with increasing disease severity. No statistically significant differences were seen in gender, number of premature infants and duration of symptoms (Table 1).

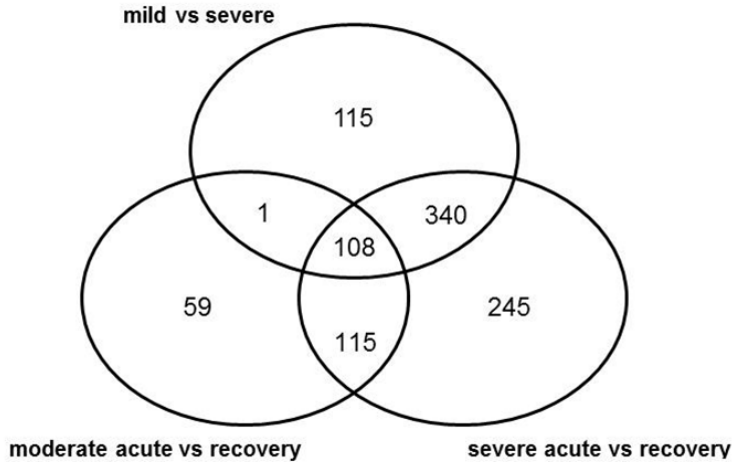

Figure 1. Venn diagram with differentially expressed genes between groups. Differentially expressed genes (q<0.05; >2 fold difference; absolute expression value >200) in patients with RSV infections comparing patients with mild vs severe disease and during acute infection vs recovery in patients with moderate and severe disease.

Microarray analyses point to OLFM4 as a marker gene to classify disease severity

The microarray analysis of PBMC of children with mild versus severe disease showed that 564 probesets were expressed differentially (428 upregulated and 136 downregulated genes) under conditions as described in material and methods (q <0.05; >2 fold difference; absolute expression value >200).

As biomarkers should discriminate between non-disease and disease, the genes expressed differentially in children with mild versus severe disease as well as during acute severe RSV infection versus recovery were selected. The analysis of paired acute and recovery samples of children with severe RSV infection resulted in 808 differentially expressed probesets (647 upregulated and 161 downregulated). Of these 808 probesets, 448 showed overlap with the 564 probesets in the comparison of mild versus severe disease, 365 genes being upregulated and 83 genes being downregulated (Figures 1 and 2).

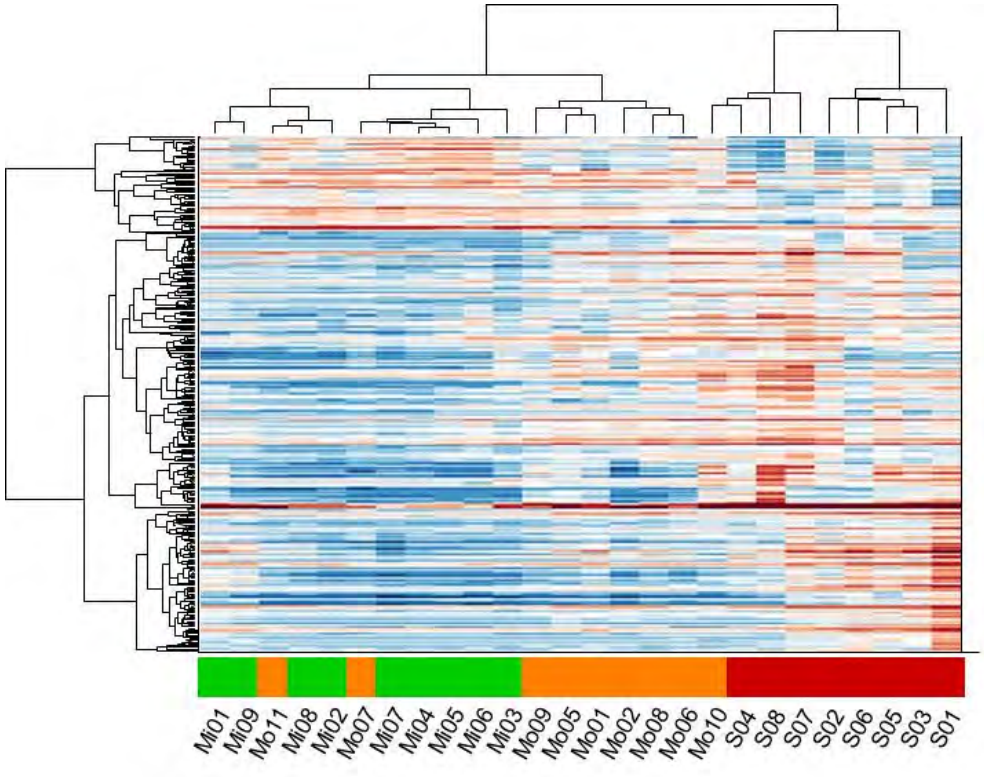

Figure 2. Discriminating mild disease from severe disease in children with RSV infection. Mi, mild; Mo, moderate; S, severe. 448 differentially expressed probesets were selected based on overlap in the comparison mild vs severe disease in RSV infected children and acute samples vs recovery samples of children with severe RSV infection. Samples were clustered based on these selected probesets by complete linkage hierarchical clustering with 1-correlation as a distance measure.

Table 2 shows the top 25 of up- and downregulated genes, of which Olfactomedin 4 (OLFM4) was the most upregulated gene with a factor of over 40 fold. Since children in the severe group were younger compared to those with mild disease, a paired age-matched subanalysis was performed among 7 severe patients versus 7 patients with mild or moderate disease.

Table 2. Top 25 up- and downregulated genes differentially expressed in PBMCs during severe RSV infection.

Upregulated genes	Fold change mild vs severe	Fold change severe acute vs recovery	Downregulated genes	Fold change mild vs severe	Fold change severe acute vs recovery
OLFM4	49.7	43.2	GNLY	-4.8	-4.0
MMP8	24.2	37.2	GZMH	-4.5	-3.2
CAECAM8	18.4	19.3	GNLY	-4.5	-3.7
ARG1	16.9	24.2	FGFBR2	-4.5	-5.1
ANXA3	16.6	16.2	TRAC	-3.9	-2.9
DEFA4	15.1	16.1	KLRF1	-3.8	-3.3
CA1	14.9	17.5	LGALS2	-3.7	-2.0
CHI3L1	13.9	11.0	KLRC1	-3.7	-3.2
LTF	13.0	12.6	KLRD1	-3.7	-3.0
SELENBP1	12.8	15.8	TRAC	-3.4	-2.8
CRISP3	12.3	12.3	THOC4	-3.4	-3.1
ELANE	11.2	10.6	GZMB	-3.4	-2.3
HP	11.0	11.9	KLRD1	-3.4	-2.7
CEACAM6	11.0	10.1	IGHM	-3.3	-2.0
HBM	10.5	26.0	GZMK	-3.3	-2.4
CHI3L1	10.3	9.2	PRF1	-3.2	-2.2
MPO	10.2	9.9	TRAC	-3.1	-2.3
ALAS2	10.2	21.7	ITPKB	-3.0	-3.5
IL1R2	10.2	10.5	SH2D1B	-3.0	-2.6
EPB42	10.1	15.3	SPON2	-3.0	-2.8
CEACAM6	10.1	9.7	PRF1	-3.0	-2.4
LCN2	10.1	10.3	TGFBR3	-2.9	-2.4
MPO	9.8	9.9	FCER1A	-2.9	-3.9
MMP9	9.8	11.6	KLRB1	-2.9	-3.9
BPI	9.6	8.9	GPR56	-2.9	-2.9

Genes that showed overlap in both the comparisons mild vs severe disease and severe acute vs recovery samples are shown

This analysis resulted in 287 differentially expressed probesets, all upregulated. The gene list of upregulated probesets did not differ substantially from the main analysis.
A supervised analysis (PAM) also identified OLFM4 as a fully discriminative marker between children with mild and severe RSV infection, giving a cross-validation error of 0%. As both SAM and PAM analyses revealed OLFM4 as a potentially important marker for disease severity in children with RSV infection and OLFM4 has -to the best of our knowledge- not been associated with respiratory tract infections before, this gene was chosen for further analysis. Interestingly, there was no marked upregulation of apoptosis genes in the severe group, indicating that the observed lymphopenia was not caused by increased apoptosis.

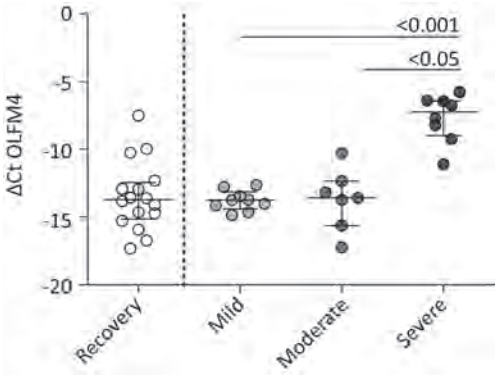

Figure 3. qPCR of patients from the microarray cohort. OLFM4 gene expression levels were significantly higher in patients with severe disease compared to those with mild and moderate disease. Expression levels are presented as ΔCt and median with inter quartile range (IQR). Statistics were performed by Kruskall Wallis tests ($p<0.001$), followed by Mann Whitney U tests for individual comparisons: mild vs moderate $p=0.26$, moderate vs severe $p<0.05$, mild vs severe $p<0.001$.

Validation of Microarray Findings by QPCR

To confirm our findings in the microarray analyses, qPCR was performed for OLFM4 in PBMCs of the same patients. One patient in the moderate group was excluded since insufficient material was left. OLFM4 expression was statistically significantly higher in patients with severe disease compared to those with mild and moderate disease (Figure 3).

OLFM4 Gene Expression in PBMCs is Increased during Acute Viral Respiratory Infection and Correlates with Disease Severity in a Validation Cohort

The validation cohort consisted of 80 children with viral LRTIs, among which 47 had a confirmed RSV infection. The characteristics of these patients differed

from those of the patients in the discovery cohort, especially for age, gender, preterm birth, duration of symptoms, and RSV Ct value (Table 3). In total, 115 PBMC samples were available for qPCR analysis, subdivided in 80 acute and 35 recovery samples. OLFM4 gene expression levels in PBMCs obtained during acute infection were higher compared to those obtained after recovery ($p<0.001$). In agreement with our microarray analyses, expression of OLFM4 in PBMCs was higher in patients with severe disease compared to those with mild and moderate disease (Figure 4). For the confirmed RSV+ patients only, OLFM4 expression also served as a discriminating marker, similar to the full validation cohort (data not shown). Length of stay (LOS) in hospital, another measure for severity, was also correlated positively with gene expression levels of OLFM4 ($\rho=0.402$, $p<0.001$).

OLFM4 Expression Correlates with Disease Severity in PBMCs but Not in Plasma

Since biomarkers in plasma are more easily obtained and processing is less time-consuming, we measured protein levels of OLFM4 in plasma of 49 randomly selected patients of the validation cohort. Although OLFM4 plasma concentrations during acute infection were statistically significantly higher compared to those in recovery samples, no association with disease severity was observed (Figure 5). No correlation between protein levels and relative gene expression was found either ($\rho=0.270$, $p=0.088$).

Table 3. Patient characteristics of validation cohort

	Mild (N=14)	Moderate (N=42)	Severe (N=24)	p-value
Age (months)	3.2 [1.1-10.3]	4.9 [2.0-14.5]	1.2 [0.6-2.8]	p<0.01*
Gender (male)	8 (57%)	22 (52%)	12 (50%)	NS
Gestational age (wks)	36.8 [39.1-40.0]	38.0 [37.0-40.0]	38.9 [37.0-40.5]	NS
Length of stay (days)	3 [2-3]	6 [4-9]	11 [10-13]	p<0.001**
Confirmed RSV infection	3 (21%)	29 (69%)	15 (63%)	<0.01***

Values are given in numbers (percentages) and median and inter quartile range (IQR). *mild versus severe p=0.05, moderate versus severe p<0.001 **mild versus moderate and severe p<0.001, moderate versus severe p<0.001 ***mild versus moderate p<0.01, mild versus severe p<0.05.

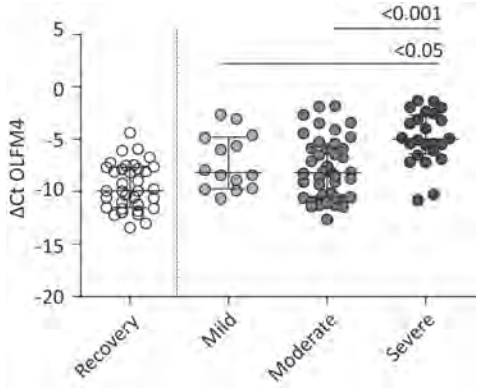

Figure 4. OLFM4 gene expression values in PBMCs from infants during acute mild, moderate and severe viral lower RTI and after recovery. Expression levels are presented as ΔCt and median with inter quartile range (IQR). Statistics were performed by Kruskall Wallis tests ($p<0.001$), followed by Mann Whitney U tests for individual comparisons: mild vs moderate $p=0.38$, moderate vs severe $p<0.001$, mild vs severe $p<0.05$.

In a Multivariable Model OLFM4 Gene Expression is a Statistically Significant Marker for Severe Disease

Relative OLMF4 gene expression, gender, gestational age, and age at time of hospital admission (in weeks) were included as determinant and potential confounders, respectively in a multivariable model for mechanical ventilation.
For OLMF4, a cut-off value of > -7.5 was chosen, which corresponds with an OLFM4 expression level greater than 0.5% that of GAPDH. The unadjusted RR of mechanical ventilation was 8.6 with a 95% confidence interval (CI) of 2.2 – 34.0. After adjustment for age and gestational age, the RR was 6.1 (95%CI: 1.5 – 24.4), which indicates that children with OLMF4 gene expression levels above -7.5 have a 6-fold increased risk of severe RSV infection requiring mechanical ventilation. Gender did not add substantially to the final model. Including OLMF4 gene expression in the model as a continuous variable resulted in an age and gestational age adjusted RR of 1.20 (95%CI: 1.04 – 1.38), meaning that the risk of receiving mechanical ventilation increased by 20% with every step increase in expression level (range -12.6 through -1.33).

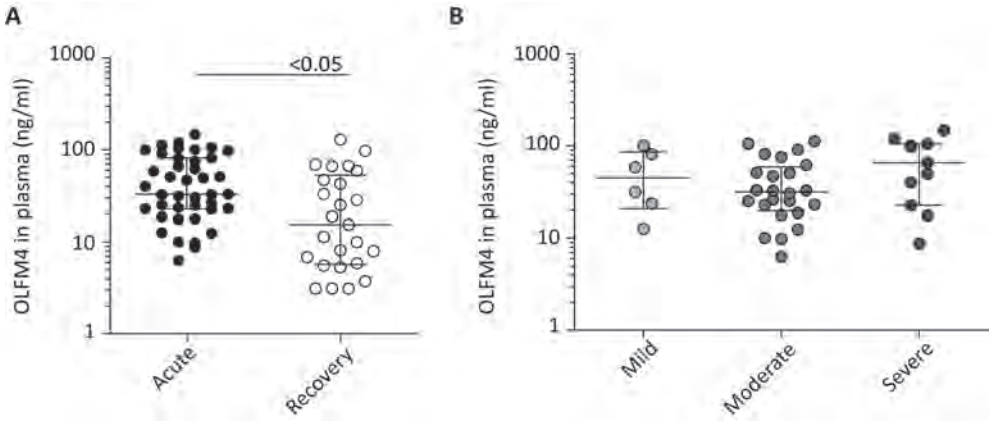

Figure 5. Plasma levels of OLFM4 in patients with viral RTI. (A) OLFM4 plasma levels are statistically significantly higher during acute (n=41) infections compared to recovery samples (n=25). (B) However, there are no statistically significant differences among the three severity groups. Plasma levels (ng/ml) are presented as median with inter quartile range (IQR). Statistics were performed by Mann Whitney U tests for comparison acute vs recovery ($p<0.05$), and Kruskall Wallis tests for comparison mild vs moderate vs severe ($p=0.29$).

DISCUSSION

In this study we demonstrated that OLFM4 gene expression in PBMC is a previously unidentified classifier for severe disease in children with viral LRTIs. OLFM4 expression was significantly increased during acute viral respiratory infections compared to recovery samples. Moreover, an association was found between OLFM4 gene expression in PBMCs and disease severity; in a multivariable model OLFM4 showed its power as a significant marker for severe disease. Children with mechanical ventilation have almost 10 times more often an increased OLFM4 expression in PBMC (greater than 0.05% of the GAPDh levels). Therefore, OLFM4 fulfills the criteria as a biomarker for disease severity, in particular to discriminate mild from severe cases in young infants. For OLFM4 to formally be used as prognostic marker, a more extensive, prospective study will be required. To the best of our knowledge, the OLFM4 gene has not been described in the context of viral respiratory infections. To test its behaviour during infectious disease we data-mined other micro-array studies that described pediatric and adult patient cohorts with infections. In a study by Ioannidis et al. (GSE 34205), we found that OLFM4 gene expression was higher in PBMCs obtained from patients with RSV (n=51) or influenza virus (n=27) infections compared to the gene expression in healthy infants (n=22), $p<0.01$ and $p<0.0001$, respectively.[31] No differences were found in OLFM4 gene expression between children under or

above three months of age with either infection by RSV or influenza. In contrast, two other studies did not observe upregulation of OLFM4 in PBMCs from children during infection by measles or rotavirus (GSE 5808 and 2729).[32, 33] Zaas et al. performed microarrays on whole blood obtained from adult volunteers at baseline and at the peak of their symptoms after being experimentally infected with RSV, influenza or rhinovirus (GSE 17156).[34] Although their data showed an upregulation of OLFM4 in RSV infected adults (p=0.01), there were no differences in OLFM4 gene expression between the baseline and during symptomatic influenza or rhinovirus infections.[34] Data of Ramilo et al. (GSE 6269-1) showed a statistically significant upregulation of OLFM4 in children, aged 0-16 years, diagnosed with influenza virus or bacterial infections (*E. coli*, *S. aureus* or *S. pneumoniae*) compared to healthy controls.[20] In this cohort, children with *S. aureus* or *S. pneumoniae* infections had statistically significantly higher OLFM4 gene expression compared to influenza A infected patients.[20]. Thus, other studies have also seen upregulation of OLFM4 expresion after bacterial or viral infection. However, none of the studies looked at disease severity.

OLFM4, also known as hGC-1 and GW112, was first cloned from G-CSF–stimulated human myeloid precursor cells and is mainly expressed in bone marrow, gastro-intestinal tract, prostate and pancreas.[35] Earlier studies have shown that OLFM4 is involved in multiple cellular functions e.g. cell growth, differentiation and apoptosis.[27] OLFM4 expression has been reported as one of several (prognostic) markers in oncology.[27] In addition, its involvement in the immune response to inflammation has been described. OLFM4 expression is upregulated in some inflammatory diseases, such as chronic inflammatory bowel diseases[36] and in Helicobacter pylori-infected patients.[37] Liu et al. showed an enhanced immune response and inflammation in OLFM4-/- mice upon Helicobacter pylori infection. Their results indicate that OLFM4 inhibits NOD1 and NOD2-mediated NF-κB activation, suggesting that OLFM4 plays an important role in regulating innate immune responses.[38] In another study, Liu et al. demonstrated that neutrophils from OLFM4 -/- mice have increased capability to kill *S. aureus* and *E. coli* and are more resistant to systemic sepsis.[39] These data suggest that OLFM4 may be an important regulator of host innate immunity against a broad array of bacterial infections. Data mining of gene expression profiling datasets (www.immgen.org) indicates that OLFM4 is besides being expressed in neutrophils, also highly expressed in Th1 cells. Therefore the increased OLFM4 expression seen in the severe subgroup may indicate an highly active ongoing Th1 response. In summary, OLFM4 was upregulated in several viral and bacterial infections in many (but not all) previously published studies investigated.

Although OLFM4 mRNA has been described to be selectively expressed in normal human myeloid lineage cells, OLFM4 protein concentrations have been measured in PBMCs, B-lymphocytes, neutrophils and monocytes.[40] This is in agreement with our results and those from the reanalyzed microarray studies, in which high and significantly different OLFM4 mRNA expression was found in PBMCs obtained from children with different severity of viral lower respiratory infections.

Clemmensen et al. showed that OLFM4 was present at protein level in only 20–25% of peripheral blood neutrophils, whereas mRNA for OLFM4 was present in all myelocytes and metamyelocytes, indicating post-transcriptional regulation as a basis for the heterogeneous expression of OLFM4 protein.[41] This may explain why we did not find differences between gene expression levels in granulocytes during acute infection and after recovery in our larger patient cohort.

In this study we did not find an association between OLFM4 plasma concentrations and disease severity. The advantage of measuring markers in plasma is the ease of implementation, speed, reproducibility and standardization. However, innovative techniques enable rapid analysis of the expression of multiple genes at transcriptional level in the near future.[42]

The severe CD4, CD8 and NK cell lymphopenia that we and others described before, can now be better explained. Together with the up regulation of activation markers on PBMC in the severe group and lack of apoptosis, the observed lymphopenia likely results from recruitment of T cells to the site of infection, i.e the lungs with an parent lower cell count in peripheral blood. In this respect peripheral blood may not reflect the situation in all parts of the body. OLFM4 is associated with Th1 responses, an active or even overaggressive Th1 response may underlie the severe clinical manifestations in this group. This is reminiscent of interpretations in the early RSV vaccine trails. Nevertheless, more accurate measurements at the site of infection will be needed to determine whether in the severe cases an inadequate adaptive immune response or a hyper responsive reaction (for instance by excessive production of cytokines) is responsible for the severe manifestations of RSV infection.

Notwithstanding this uncertainty, in this study we are the first to show that OLFM4 transcription is associated with severity of disease in children with viral lower respiratory tract infections, also after correcting for age. These results emphasize the role of OLFM4 in innate and adaptive immunity and encourages further research into the presence of OLFM4 in PBMCs and the pathogenesis of RSV infections. Moreover, it could lead to the development of a new diagnostic tool to predict a severe course of viral respiratory disease and aid the physician in clinical decisions.

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Chapter 7

Quantitative proteome profiling of respiratory virus-infected lung epithelial cells

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ABSTRACT

Background

Respiratory virus infections are among the primary causes of morbidity and mortality in humans. Influenza virus, respiratory syncytial virus (RSV), parainfluenza (PIV) and human metapneumovirus (hMPV) are major causes of respiratory illness in humans. Especially young children and the elderly are susceptible to infections with these viruses. In this study we aim to gain detailed insight into the molecular pathogenesis of respiratory virus infections by studying the protein expression profiles of infected lung epithelial cells.

Methods

A549 cells were exposed to a set of respiratory viruses (RSV, hMPV, PIV and Measles virus (MV)) using both live and UV-inactivated virus preparations. Cells were harvested at different time points after infection and processed for proteomics analysis by 2-dimensional difference gel electrophoresis (2D-DIGE). Samples derived from infected cells were compared to mock-infected cells to identify proteins that are differentially expressed due to infection.

Results

We show that RSV, hMPV, PIV3, and MV induced similar core host responses and that mainly proteins involved in defense against endoplasmatic reticulum (ER) stress and apoptosis were affected which points towards an induction of apoptosis upon infection.

Conclusion

By 2-D DIGE analyses we have gathered information on the induction of apoptosis by respiratory viruses in A549 cells.

INTRODUCTION

Respiratory viruses are a major cause of lower respiratory tract infections (LRTIs) and are among the primary causes of morbidity and mortality in humans. These infections are a major public health problem. Especially infants and young children as well as the elderly are prone to severe and even fatal outcome of infections with respiratory viruses such as Influenza virus, RSV, PIV, and human hMPV.[1-5] In young children and infants, RSV is the primary etiologic agent of epidemic lower respiratory tract infections being responsible for 80% of cases of acute bronchiolitis.[6, 7] For RSV extensive vaccine-related research has been performed, but so far no efficient and widely applicable vaccine has been developed yet. Also for the recently discovered hMPV[8], which is closely related to RSV, no vaccine is currently available. Only for MV an effective vaccine is available and for classical Influenza vaccines are available but these need further improvement.

RSV, hMPV, PIV and MV belong to the Paramyxoviridae family of viruses These are all enveloped viruses and have a linear, non-segmented, single stranded negative sense RNA of ~15,000 nucleotides containing 10 genes encoding 10 (RSV) proteins, 13 kb containing 8 genes (hMPV), ~15,500 nucleotides containing 6 genes (PIV), and 15.9 kb containing 6 genes (MV), respectively.[9] All these viruses enter the host via the respiratory tract by infecting airway epithelial cells that line the nose as well as the large and small airways and induce inflammation associated with the disease at the site of infection with the exception of MV that disseminates beyond the respiratory tract.[9]

Although respiratory cells are the first targets of respiratory virus infection, they are also the first line of defense in the innate immune response that is generated upon infection.[10] Epithelial cells form a direct physical barrier between the host and the environment, the epithelial cells are able to detect invading pathogens and actively participate in the innate immune response to invading viruses e.g. by producing acute phase proteins and inflammatory cytokines and chemokines.[10-12] Depending on the location in the lung, there are different types of epithelial cells that respond to invading pathogens in a cell type specific manner due to varying expression of pattern recognition receptors, cell-specific protein expression, or to differences in susceptibility to injury. These responses can result in killing of the pathogen by recruitment of phagocytes that can not only directly kill the invading pathogens but also shape the adaptive immune response to viral infection

However, little is known about the exact mechanisms underlying the induction and maturation stages of innate and adaptive anti-viral immune responses.

Also the interaction between the virus and the host at the molecular level is not well understood. A better understanding of these events is required for the development of new strategies for treatment and prevention of these infectious diseases. In this study we describe the effect of respiratory virus infection (RSV, hMPV, PIV and MV) on protein expression levels in type II alveolar A549 human epithelial cells by 2-D DIGE analyses at different time points after infection to gain detailed insight into the interaction between the viruses and the host cells and to explore differences in induced protein expression changes between different respiratory viruses. A549 cells are frequently used for in vitro infection with RSV and all four viruses are capable of infecting these cells making it a suitable in vitro model for comparing protein expression profiles between RSV, hMPV, PIV, and MV.[12-17] We show that a similar core host response was induced by the different viruses that were studied, and that RSV, hMPV, PIV3, and MV mainly affect proteins involved in defense against ER stress and apoptosis which points towards an induction of apoptosis upon infection.

MATERIALS AND METHODS

Chemicals and Reagents

Fluorescent minimal labeling CyDyes and IPG buffer pH3–10 were purchased from GE Healthcare (Roosendaal, The Netherlands). Urea, thiourea, Tris, iodoacetamide, trifluoroacetic acid (TFA), phosphoric acid, acetone, glycerol, acetonitrile, ammonium bicarbonate, CHAPS, l-lysine, and porcine modified trypsin were all from Merck (Amsterdam, The Netherlands), DTT from Sigma-Aldrich (Zwijndrecht, The Netherlands), DHS from Fluka, Trizol from Invitrogen (Breda, The Netherlands), TEMED, low melting temperature agarose (LMT), ammoniumpersulphate and Coomassie Brilliant Blue R-250 from BHD (Amsterdam, The Netherlands), and acrylamide from Nation Diagnostics (Hessle, UK).

Cells and Viruses

The human lung adenocarcinoma epithelial cell line A549 and (American Type Culture Collection (ATCC; CCL-185), Manassas, VA, USA) and the Vero-118 cell line (a gift from B.G. van den Hoogen) were cultured at 37 °C with 5% CO2 in respectively HAM F12 medium (GIBCO) supplemented with 3% heat-inactivated fetal calf serum (FCS, Hyclone) and Iscove's Modified Dulbecco's Medium (IMDM,

GIBCO) supplemented with either 0.02% trypsin and 3% bovine albumin fraction V medium (for hMPV infection) or 10% FCS (for RSV, PIV, and MV infections). All media were also supplemented with penicillin (100 U/ml; BioWhittaker), streptomycin (100 µg/ml; BioWhittaker) and l-glutamin (2 mM; BioWhittaker). High titer stocks from hMPV (NL/1/00, a gift from B.G. van den Hoogen), RSV-A2 (ATCC; VR1540), PIV3 (ATCC; VR-93), and MV-Edm (ATCC; VR-24) were grown on Vero-118 cells using a serial limiting dilution protocol according to Gupta et al. and used to infect A549 cells.[18] From all virus stocks inactivated virus preparations were produced by UV-irradiation using an Uvitec ultraviolet transilluminator (312 nm wavelength).

In Vitro Infection Experiments

A549 cells were seeded at a density of 3×10^5 cells/well in a 6-well plate and were allowed to adhere overnight. Live or UV-inactivated virus preparations (same dilution from the original high tittered stocks) were added to the cells at a multiplicity of infection of 3 to 10. At 60 min after infection the virus-containing serum free medium was removed and replaced by a fresh HAM F12 medium (containing 3% FCS) and cells were incubated at 37 ˚C. For the mock-infected cells a medium containing no virus was added to the cells. At 6, 12, and 24h after infection the cells were washed once with pre-warmed PBS and the cells were lysed in 1 ml Trizol and stored at -80 ˚C. These infection experiments were performed in duplicate on different days for analysis of biological replicates.

Protein Isolation

Proteins were isolated from the remaining interphase and organic phase that remain after RNA extraction from Trizol samples. Four volumes of ice-cold (-20 ˚C) acetone were added to these fractions and incubated at -20 ˚C for 1 h. Precipitated proteins were then centrifuged at maximum speed for 5 min. The protein pellet was washed twice with ice-cold 80% acetone. The pellet was air-dried and suspended in milliQ. Protein concentration in each sample was determined using a 96-well plate based BCA assay (Pierce) according to the manufacturer's instructions.

Protein Labeling

A total amount of 25 µg protein was taken from each sample and dried in a

vacuum concentrator. The protein pellets were dissolved in 10 µl lysis buffer (30 mM Tris, 7 M urea, 1 M thiourea, and 4% CHAPS) and were snap frozen five times to completely dissolve the protein pellet. Per 25 µg protein 200 pmol fluorescent minimal labeling CyDye was added according to the scheme depicted in Table 1 and incubated on ice for 30 min in the dark. 1 µl 10 mM l-lysine was added to each sample and was incubated for 15 min in the dark to stop the labeling reaction. Labeled samples were stored at -20 ˚ until use.

Table 1. Experimental setup for 2-D DIGE experiments[1].

Gel number	Cy3	Cy5	Cy2
1–2	Mock t = 0	Mock t = 6	A549 mock
3–4	Mock t = 12	Mock t = 24	A549 mock
5–6	RSV + UV t = 6	RSV - UV t = 6	A549 mock
7–8	RSV + UV t = 12	RSV - UV t = 12	A549 mock
9–10	RSV + UV t = 24	RSV - UV t = 24	A549 mock
11–12	hMPV + UV t = 6	hMPV - UV t = 6	A549 mock
13–14	hMPV + UV t = 12	hMPV - UV t = 12	A549 mock
15–16	hMPV + UV t = 24	hMPV - UV t = 24	A549 mock
17–18	PIV + UV t = 6	PIV - UV t = 6	A549 mock
19–20	PIV + UV t = 12	PIV - UV t = 12	A549 mock
21–22	PIV + UV t = 24	PIV - UV t = 24	A549 mock
23–24	MV + UV t = 6	MV - UV t = 6	A549 mock
25–26	MV + UV t = 12	MV - UV t = 12	A549 mock
27–28	MV + UV t = 24	MV - UV t = 24	A549 mock

[1]50 µg protein from each sample was labeled with fluorescent CyDyes as indicated.

2-D Gel Electrophoresis

Samples to be run on the same gel were combined (Table 1) and used for rehydration of 18 cm Immobiline Dry Strips pH4–7 (GE Healthcare). A volume of 310 µl rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1.2% IPG buffer pH3–10, and 2 mg/ml DTT) was added to the combined samples and applied to the strips using a rehydration tray. After an overnight rehydration the proteins were separated in the first dimension using a Multiphor II (3 h 300 V followed by a gradual increase by 300 V every 20 min until a final voltage of 2200 was reached). After complete focusing (± 30 kVh), the strips were equilibrated in 0.5% DTT in equilibration buffer (100 mM Tris pH8.8, 6 M urea, 30% glycerol, and 2% SDS) for 10 min followed by 4.5% iodoacetamide in equilibration buffer

for 10 min. The strips were then applied to a 12–20% gradient polyacrylamide gel that had been made the day before using low-fluorescent glass plates. The strips were fixed using a 1% LMT solution and the proteins were directly separated overnight in the second dimension using an Ettan Dalt Six (GE Healthcare) at 10 °C and 2 W per gel.

Scanning and Image Analysis

Gels were scanned on a Typhoon 9410 (Amersham Biosciences) at excitation wavelengths of 488, 532, and 633 nm and 520BP40, 580BP30, and 670BP30 emission filters Cy2, Cy3, and Cy5, respectively. For the comparative analyses per time point only those protein spots were included that were present in all of the gels to be compared for a certain virus or time point. Statistical analysis and quantitative protein expression were done using Decyder Analysis BVA software (Amersham Biosciences) and protein spots were considered significantly differential at a p-value of < 0.01 and a fold change of at least 2.5.

Mass Spectrometry and Protein Identification

For identification of protein spots a preparative gel containing 500 µg protein was run as described earlier and stained with Coomassie Brilliant Blue R-250. Protein spots of interest were manually excised from the gel and digested with trypsin. Peptides were extracted using 2% TFA and were desalted and concentrated using C18 StageTips. Peptide mixtures were purified and desalted using C18 StageTips. Peptide separation and sequence determination were performed with a nano-high performance liquid chromatography system (Agilent 1100 series, Amstelveen, The Netherlands) connected to a 7-T linear quadrupole ion trap-ion cyclotron resonance Fourier transform mass spectrometer (Thermo Electron, Breda, The Netherlands). Peptides were separated on a 15-cm 100-µm-inner-diameter PicoTip emitter for online electrospray (New Objective, Woburn, MA) packed with 3 µm C18 beads (Reprosil, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) with a 60-minute linear gradient from 2.4 to 40% acetonitrile in 0.5% acetic acid at a 300 nl/min flow rate. The four most abundant ions were sequentially isolated and fragmented in the linear ion trap by applying collisionally induced dissociation. Proteins were identified using the MASCOT search engine (Matrix science, London, UK) against the human protein NCBI database using the following search criteria: 20 ppm peptide tolerance, a maximum of 2 missed cleavages, a fixed carbamidomethyl modification of cysteines, and variable oxidation (M) and

deamidation (NQ) modification.

Molecular Network Analyses

The global protein–protein interaction (PPI) network used (herein referred to as galaxy 6) was built from an accumulation of human-curated PPIs obtained from the Biomolecular Interaction Network Database (BIND) (data downloaded in October 2006), the Human Protein Reference Database (HPRD) (data of release 6th of January 2007), the IntAct database (downloaded in May 2007), the Molecular Interactions Database (MINT) (downloaded in May 2007), and the PDZBase database (downloaded in May 2007). All PPI networks were drawn using Cytoscape. The Gene Ontology (GO) database used was downloaded from NCBI in February 2008. Gene enrichment analyses were done using BINGO.[19] Protein enrichment analyses were done using in-house python scripts.

RESULTS AND DISCUSSION

2-D DIGE analyses

To study the epithelial responses upon respiratory virus encounter and infection, A549 cells were exposed to different respiratory viruses (RSV, hMPV, PIV and MV). Both live and UV-inactivated virus preparations were used thus allowing distinction between live, replicating, virus-induced changes in protein expression and changes induced upon contact with or uptake of non-replicating virus particles. At different time points after infection, the cells were lysed and used for protein expression analysis by 2-D DIGE and quantitative and comparative DeCyder analyses. Approximately 1000 spots were detected in the individual gels. Of these, 325 were present in at least 20 out of 28 gels and could be used for statistical and comparative analyses. For the comparative analyses per time point only those protein spots were included that were present in all of the gels to be compared for a certain virus or time point. Upon infection with either one of the respiratory viruses or mock, a total of 70 spots showed differential expression in at least one of the time points after infection (Fig. 1A). Differentially expressed spots were manually excised from a preparative gel and were analyzed by nano-LC FT-ICR-MS to identify the proteins. Of the 70 spots that were picked for analysis we were able to identify the corresponding protein in 63 samples.

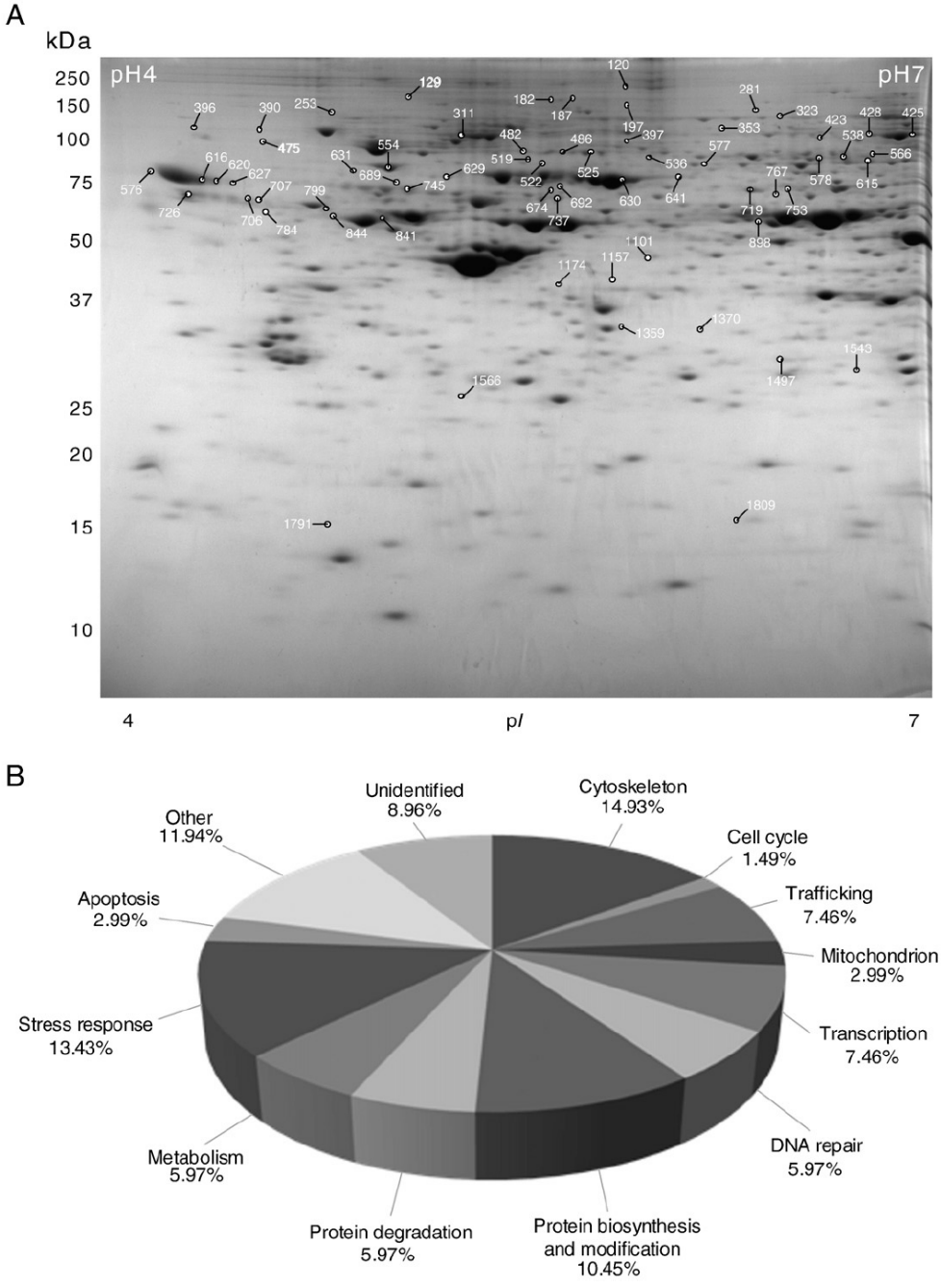

Figure 1. Preparative gel image (A) and functional classification (B) of protein spots that showed differential expression upon respiratory virus infection of A549 cells compared to mock at 6, 12 or 24 h after infection.

Table 2. Differentially expressed spots

Estimated					FT-MS analysis			RSV[a, b]			hMPV[a, b]			PIV[a, b]			MV[a, b]		
Spot nr.	Mass (Da)	pI	Protein ID	protein description	gene	# pept	Seq. cov. (%)	protein score	6	12	24	6	12	24	6	12	24	6	12	24
Cytoskeleton/ structural proteins																				
311	125000	5.30	O43707	alpha-actinine 4	ACTN4	26	34.9	1021						-2.65, -3.25							
323	150500	6.48	P18206	vinculin	VCL	6	5.6	187			-4.39, -2.54										
390	115000	4.53	O43707	alpha-actinine 4	ACTN4	12	12.5	431									-2.84,-				
396	110000	4.35	O43707	alpha-actinine 4	ACTN4	13	14.4	410										-2.81,-	-,- 6.51		
577	95000	6.20	P02545	lamin A/C	LMNA	20	32.4	828						-8.46							
578	97500	6.60	P35241	radixin	RDX	48	75.8	1545	-, 11.12		-,2.14				2.82,-				3.82,-		
615	97500	6.80	P02545	lamin A/C	LMNA	33	51.2	1143							2.41,-						
629	80000	5.25	P20700	lamin B1	LMNB1	37	55.5	1313						-4.22, -6.17							
841	52000	5.00	Q13509	tubulin beta-3 chain	TUBB3	16	40.9	767					3.08, 2.38								
844	53000	4.85	P07437	tubulin beta-2 chain	TUBB	5	14.6	241		4.69, 4.70											
Cell cycle																				
129	180000	5.10	Q14978	nucleolar phosphoprotein p130	NOLC1	3	3.6	95										-3.75,-			
Vesicular trafficking/ transport																				
182	190000	5.58	Q15075	early endosome antigen 1	EEA1	9	5.2	362						-3.69,-							
187	190000	5.65	Q15075	early endosome antigen 1	EEA1	2	1.3	72						-5.22,-	2.17,-						
423	125000	6.60	Q05193	dynamin-1	DNM1	28	28.6	943										-2.04,-			3.54,-
674	75000	5.60	Q96HE7	ERO-like protein alpha (precursor)	ERO1L	11	26.1	378													-7.68,-
689	77500	5.08	Q9UNF0	protein kinase C and casein kinase substrate in neurons protein 2	PACSIN2	12	23.0	413								-,-3.78					
Mitochondrion																				
522	95000	5.55	P28331	NADH-ubiquinone oxido-reductase 75kDa subunit																	
1157	41000	5.85	Q9HB07	MYG1 protein																	
Transcription/ RNA processing																				
197	175000	5.83	Q00839	heterogeneous ribonu-cleoprotein U	HNRPU	1	1.0	36	-,9.11					-2.08,-							

	Estimated				FT-MS analysis			RSV[a, b]			hMPV[a, b]			PIV[a, b]			MV[a, b]		
Spot nr.	Mass (Da)	pI	Protein ID	protein description	gene	# pept	Seq. cov. (%)	protein score	6	12	24	6	12	24	6	12	24	6	12	24
253	150000	4.88	Q15029	116 kDa U5 small ribonucleoprotein U	EFTUD2	5	5.5	188						-2.76,-						
311	125000	5.30	P19338	nucleolin	NCL	21	24.1	923						-2.65, -3.25						
566	100000	6.83	Q8TCS8	polyribonucleotide nucleotidyltransferase 1	PNPT1	43	54.0	1336									10.85, 13.72			
745	70000	5.13	P61978	heterogeneous ribonucleoprotein K	HNRPK	10	23.8	412	3.71,-											
DNA repair and maintenance																				
482	105000	5.50	P49959	double strand break repair protein MRE11A	MRE11A	5	6.8	219							5.04, 5.41					7.06,-
486	105000	5.63	P13010	ATP-dependent DNA helicase II, 80kDa subunit	XRCC5	6	8.7	258	3.29, 3.70											
525	105000	5.75	P13010	ATP-dependent DNA helicase II, 80kDa subunit	XRCC5	27	51.6	1061	2.52, 2.44											
799	60000	4.85	P54727	UV excision repair protein RAD23 homologB	RAD23B	14	34.0	436				4.33,-								
Protein biosynthesis and modification																				
425	130000	6.98	P13639	elongation factor 2	EEF2	44	46.6	1646						-2.62,-						
428	130000	6.80	P13639	elongation factor 2	EEF2	22	25.3	785			-4.37, -4.24									
576	80000	4.08	P41250	glycyl-tRNA synthetase	GARS	2	2.8	86			-,- 2.99									
577	95000	6.20	P41250	glycyl-tRNA synthetase	GARS	27	34.8	885						-,8.46						
707	63000	4.58	P07237	protein disulfide-isomerase precursor	P4HB	2	4.1	85			-,- 2.50	2.13,-			2.55,-					
1101	44500	6.00	Q9Y570	protein phophatase methylesterase 1	PPME1	9	26.7	345	-,2.90											
1497	28500	6.48	O15305	phosphomannomutase 2	PMM2	13	49.2	442						-2.71,-						
Protein degradation																				
311	125000	5.30	P55072	transitional endoplasmatic reticulum ATPase	VCP	20	27.9	710						-2.65, -3.25						
311	125000	5.30	Q9UBT2	SUMO activating enzyme subunit 1	SAE2	14	18.4	476						-2.65, -3.25						
692	75000	5.65	Q9H4A4	aminopeptidase B	RNPEP	19	36.9	689												-3.03,-
1566	24500	5.30	P61086	ubiquitin-conjugating enzyme E2-25 kDa	HIP2	6	31.5	187												5.19,-
Metabolism																				
538	100000	6.70	Q06210	glucosamine-fructose-6-phosphate aminotransferase	GFPT1	12	16.5	376									5.62, 4.88		3.48,-	

Table 2. (*continued*)

	Estimated					FT-MS analysis			RSV[a, b]			hMPV[a, b]			PIV[a, b]			MV[a, b]		
Spot nr.	Mass (Da)	pI	Protein ID	protein description	gene	# pept	Seq. cov. (%)	protein score	6	12	24	6	12	24	6	12	24	6	12	24
753	75000	6.48	P31939	bifunctional purine bio-synthesis protein PURH	ATIC	6	10.3	181									-2.42, -2.74			
898	57500	6.38	P30838	aldehyde dehydrogenase, dimeric NADP-preferrin	ALDH3A1	9	17.5	308												-,2.13
1543	27000	6.70	P60174	triphosphate isomerase	TPI1	2	10.0	74									3.04, 2.70			
Stress respnse/ molecular chaperones																				
397	125000	5.90	P07900	heat shock protein 90-alpha	HSP90AA1	26	33.5	1056					4.48, 5.95							
519	97500	5.53	P11142	heat shock cognate 71 kDa protein	HSPA8	4	6.3	130								-,5.83				
554	90000	5.05	P11021	78 kDa glucose regulated protein (precursor)	HSPA5	47	77.5	2001						-2.23, -3.01						
631	80000	4.95	P11142	heat shock cognate 71 kDa protein	HSPA8	15	25.4	471								-,-2.89				
719	77500	6.35	P49368	T-complex protein1, gamma subunit	CCT3	38	69.0	1292									-2.38,-			
726	63000	4.30	P49368	T-complex protein1, gamma subunit	CCT3	7	9.7	219										-4.32,-		
737	70000	5.63	P48643	T-complex protein1, epsilon subunit	CCT5	38	71.0	1300						-2.15, -2.46						
767	72500	6.45	P40227	T-complex protein1, zetasubunit	CCT6A	16	25.6	569									-4.90, -2.91			
1809	17000	6.30	P02763	alpha-1-acid glycoprotein 1 (precursor)	ORM1	5	27.4	172											-,6.85	
Apoptosis																				
353	135000	6.30	Q8WUM4	programmed cell death 6-interacting protein	PDCD6IP	31	39.1	1297			-3.24, -2.40			-3.29,-						-2.43,-
1791	17000	4.88	Q15121	astrocytic phophoprotein PEA-15	PEA15	2	18.5	81										-2.68,-		
Other/ unknown function																				
616	74000	5.33	P02765	alpha-2-HS-glycoprotein (precursor) (Fetuin-A)	AHSG	6	11.4	201			-, -3.00									
620	72000	4.43	P02765	alpha-2-HS-glycoprotein (precursor) (Fetuin-A)	AHSG	5	8.7	185			-, -2.96									
627	72000	4.50	P02765	alpha-2-HS-glycoprotein (precursor) (Fetuin-A)	AHSG	5	8.7	168			-, -3.56									
641	85000	6.13	O95671	N-acetylserotonin O-met-hyltransferase like	ASMTL	13	24.3	456			3.23, 3.10									

	Estimated				FT-MS analysis				RSV[a, b]			hMPV[a, b]			PIV[a, b]			MV[a, b]		
Spot nr.	Mass (Da)	pI	Protein ID	protein description	gene	# pept	Seq. cov. (%)	protein score	6	12	24	6	12	24	6	12	24	6	12	24
706	63000	4.53	P02765	alpha-2-HS-glycoprotein (precursor) (Fetuin-A)	AHSG	5	8.7	156			-, -2.66									
784	55000	4.63	Q9H8Y8	golgi reassembly stacking protein 2	GORASP4	2	2.8	62									2.81, -5.23			
1174	40000	5.60	P06748	nucleophosmin	NPM1	5	23.5	158									4.90, 3.31			
Unidentified																				
120	N/A	N/A	N/A	N/A	N/A				-, 13.75						2.35, -					
174	N/A	N/A	N/A	N/A	N/A												-, 2.34			
281	N/A	N/A	N/A	N/A	N/A														-, 2.43	
475	N/A	N/A	N/A	N/A	N/A							-, 2.63								
630	N/A	N/A	N/A	N/A	N/A					2.57, 3.14										
1359	N/A	N/A	N/A	N/A	N/A						2.99, 2.02									
1370	N/A	N/A	N/A	N/A	N/A															-, 6.85

[a,b]Numbers indicate fold changes in protein expression upon infection with live (a) and UV-inactivated (b) virus particles compared to mock-infected cells.
Only significant changes are shown
-: not significantly changed

This resulted in the positive identification of 55 unique proteins (Table 2). These proteins were shown to be mostly involved in cellular structures, stress responses, protein biosynthesis and modification, transcription, and trafficking (Fig. 1B).

Respiratory Virus-Induced Changes in Protein Expression

Upon infection of A549 cells with the 4 different respiratory viruses, there were 21, 18, 19 and 17 spots that showed differential expression upon infection with RSV, hMPV, PIV or MV, respectively, compared to mock-infected cells at 6, 12 or 24 h after infection (Table 2). RSV, hMPV, PIV and MV belong to the family of the Paramyxoviridae and are related to each other yet inducing distinct disease phenotypes. What became apparent from the 2-D DIGE analyses is that RSV and hMPV infected cells showed slightly more induced changes in protein expression than the PIV and Measles virus-infected cells. Also no clear differences were found in direct comparisons between protein samples from live and UV-inactivated virus-infected cells, indicative for a predominating general response upon virus encounter by A549 cells with no apparent effect of the replication on the proteome within the time frame of the experiment. In addition, only proteins with a high turn-over rate will show differential expression at earlier time points.

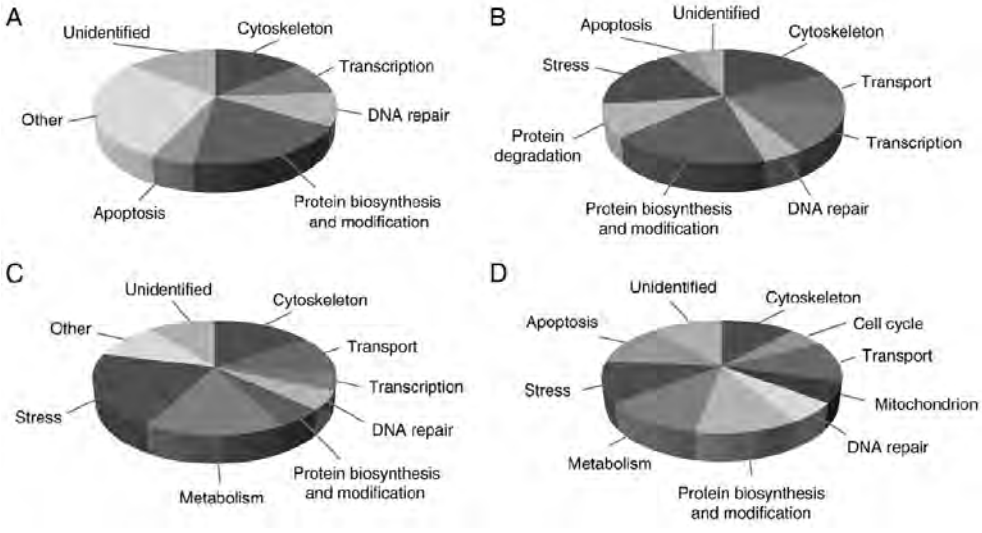

Figure 2. Functional classification of proteins that showed differential expression upon infection of A549 cells with respiratory viruses RSV (A), hMPV (B), PIV (C), and MV (D).

Table 3. Molecular and cellular functions of differentially expressed proteins.

Molecular and cellular functions	Number of proteins[1]				
	All	RSV	hMPV	PIV	MV
Cellular organization, morphology, function and proliferation	26	8	12	13	11
Cell death	22	7	14	10	10
Metabolism	11	4	5	6	7
Small molecule biochemistry	14	4	6	4	6
Molecular transport	8	3	5	4	5
Post-translational modification and folding	8	2	5	5	4
Cellular compromise	3	3	5	4	1
DNA replication, recombination, and repair	6	2	5	3	2
Cell-to-cell signaling and interaction	5	3	3	2	2
Gene expression	5	2		3	2
Protein synthesis	4	2	3	1	1
Cellular development	2		2	3	
Infection mechanism	3	1	2		2
Cell signaling	1	1	3		
Cell-mediated immune response	4		4		
Protein Degradation	3		1		3
RNA post-transcriptional modification	3		1	1	1
RNA trafficking	1	1	1		1
Inflammatory response	3	1			1
Cellular movement	1	1			1
RNA damage and repair	2		1	1	
Cellular response to therapeutics	1	1			
Energy production	1				1
Free radical scavenging	1				1

[1]Only significant changes are shown.

An additional caveat regarding a non-ambiguous interpretation of the differential protein responses measured by 2-D DIGE is an accumulation of virus-induced changes in addition to host cellular defense responses upon encounter with the virus particles. Given the observation that there were not many differences between live and UV-inactivated virus particles, the most likely effect is that of the cell upon encounter with the virus particles instead of the activity of the live replicating viruses. Upon infection of A549 cells with RSV, eleven proteins were more abundant, 9 less abundant, and 1 more abundant at 6 h while less abundant at 24 h compared to mock-infected cells (Table 2). Most of these

proteins are known to be involved in protein biosynthesis and modification, and cellular structures (Table 2 and Fig. 2A). A total of 18 protein spots were differentially expressed in A549 cells upon encounter with hMPV, of which 7 were more abundant and 11 less abundant (Table 2 and Fig. 2B). A major part of the differentially expressed proteins are known to be involved in protein biosynthesis and modification, cellular stress responses, transcription or are cytoskeleton- or structural proteins. PIV infection showed more abundant expression in 12 protein spots, less abundant expression in 6 spots and 1 spot that was more abundant in live virus particles infected cells while less abundant in UV-inactivated virus-infected cells (Table 2 and Fig. 2C). Finally, MV showed less abundant expression in 8 spots and more abundant expression in 9 spots (Table 2 and Fig. 2D).

In addition to functional annotations, we also used Ingenuity for functional analyses on these differentially expressed proteins and identified 34 molecular and cellular functions for these proteins. Most functions include cell death, cellular organization and function and morphology, post-translational modifications and folding, and metabolism (Table 3, Fig. 1B).

Table 4. GO enrichment analyses of respiratory virus altered proteins in A549 cells.

Case	Virus-perturbed	Random input	Virus-perturbed recovered	Average random recovered	Fold enrichment	p-value[1]
RSV tx in original network	8	8	7	1.43	4.89	< 0.001
RSV tx in apoptosis network	8	8	1	0.14	7.25	0.007
RSV tx apoptosis enriched	8	8	8	0.47	16.88	< 0.001
RSV tx apoptosis-cytokine enriched	8	8	3	0.47	6.33	0.002
hMPV tx in original network	17	17	11	3.11	3.54	< 0.001
hMPV tx in apoptosis network	17	17	3	0.27	11.07	< 0.001
hMPV tx apoptosis enriched	17	17	17	1.05	16.19	< 0.001
hMPV tx apoptosis-cytokine enriched	17	17	8	1.05	7.62	< 0.001
PIV tx in original network	14	14	10	2.53	3.95	< 0.001
PIV tx in apoptosis network	14	14	1	0.23	4.39	0.013
PIV tx apoptosis enriched	14	14	14	0.85	16.45	< 0.001
PIV tx apoptosis-cytokine enriched	14	14	6	0.85	7.05	< 0.001
MV tx in original network	12	12	7	2.15	3.25	0.001
MV tx in apoptosis network	12	12	2	0.20	10.20	0.001
MV tx apoptosis enriched	12	12	12	0.72	16.55	< 0.001
MV tx apoptosis-cytokine enriched	12	12	3	0.72	4.14	0.005

[1]P-value refers to how likely random recovered number is higher than virus-perturbed recovered.

These analyses also showed that all 4 viruses affect these cellular processes similarly (Table 3).

Molecular Network Analyses

Results from the original GO network analysis (Fig. 3, arrow 1, result 1) show that many of the identified proteins (viral perturbed) are involved in apoptosis. To further explore the involvement of respiratory virus-induced or repressed proteins in the apoptosis pathway we performed a GO enrichment analysis as schematically depicted in Fig. 3A (arrows 2, 2a, and 3, result 2). The apoptosis network was extracted from the original GO network and was enriched for proteins known to interact with proteins involved in apoptosis resulting in the apoptosis N1 protein network (arrow 2b, Fig. 3A). When virus-perturbed proteins were analyzed using this database 100% of the proteins were recovered (arrow 4, result 3), thus indicating the involvement of these proteins in apoptosis, either directly or indirectly by interacting with proteins that are directly involved in apoptosis (Table 4).
Since cytokines have been shown to be of great importance in respiratory virus infection and are known to be involved in apoptosis as well, we generated a new network from the apoptosis N1 network and the cytokine network from the original GO database together with the virus-perturbed proteins (cytokine and virus-perturbed network) and extracted the virus-perturbed N1 network (arrow 6, result 4). Analysis of the virus-perturbed proteins in this database revealed that 25–55% of these proteins are either proteins involved in apoptosis themselves or cytokines known to interact with these apoptosis proteins (Fig. 3B).
A substantial part of the proteins that were differentially expressed upon infection with either one of the different viruses are involved in apoptosis. To check whether this observation was not based on coincidence we performed another GO enrichment procedure for randomly selected proteins. Table 4 shows that the number of recovered proteins was significantly higher in the virus-perturbed than for the randomly selected proteins, indicating that the proteins that were found differentially expressed are indeed mostly involved in apoptosis.

Functional Pathway Analyses

Ingenuity functional pathway analyses showed that a total number of 22 differentially expressed protein spots is considered to be involved in cell death and 26 in cellular organization, function and proliferation (Table 3). Ingenuity

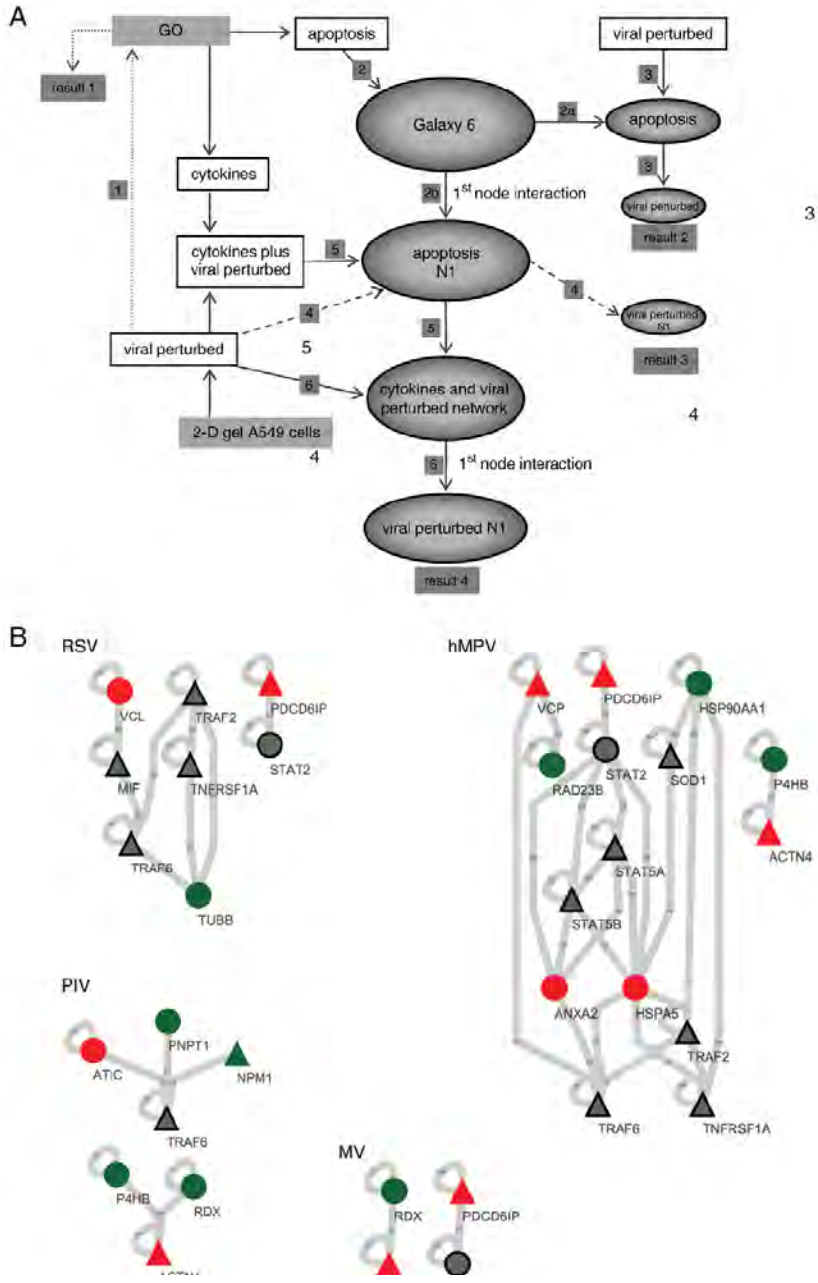

Figure 3. GO enrichment analysis procedure (A) and protein-protein interactions (B) of differentially expressed proteins in respiratory virus-infected A549 cells. The numbers on arrows in figure A represent the order in which the GO enrichment procedure was performed. Triangles in figure B represent proteins involved in apoptosis, nodes with dark bold borders are cytokines. Green nodes are proteins that were more abundant upon infection, while red nodes represent less abundant proteins.

function analyses revealed that there is a great overlap between these molecular and cellular functions, since similar proteins were categorized in these two functional categories. Both categories include VCL, RDX, HSPA5, T-complex protein 1, HSPA90AA1, NDUFS1, NCL, PNPT1, XRCC5, ALDH3A1, PDCD6IP, and NPM1 and proteins that are cell death associated only LMNB1, TUBB3, RAD23B, EEF2, P4HB, VCP, HSPA8, and PEA15 (Table 5). The process of virus-induced apoptosis can be divided into 4 processes based on these proteins, namely, virus uptake and infection, stress response, disruption of cellular structures and cell death by apoptosis.

Virus Uptake and Infection

XRCC5 is the 86 kDa subunit of the Ku complex that is abundant in the nucleus and binds to DNA.[20] Ku was postulated to have a role in DNA repair or replication as it has been shown to bind to the termini of DNA fragments. It was shown previously that Ku86 has a role in HIV1 and SV40 infection and our data suggest a role for this protein in RSV infection of A549 cells as well.[21-23]
HSPA8 is a member of the HSP70 family of heat shock proteins and has been shown to be virus inducible showing highest expression during the S-phase of cell cycle and is necessary for the entry of HTLV-I into its target cells.[24, 25] In hMPV and PIV infected A549 cells, HSPA8 is more abundant at 6 and 12 h respectively. This shows that hMPV is inducing expression in A549 cells early in infection while this response is somewhat slower in PIV infected cells.

Stress Response Proteins

Of the set of differentially expressed proteins described above, HSPA5, HSP90AA1, TUBB3, P4HB and VCP are all proteins that are involved in cellular stress responses preceding apoptosis. All these proteins were shown to be less abundant at 24 h after infection except for TUBB3, a member of the tubulin family of microtubule proteins that is also known to be involved in adaptation to oxidative stress and cellular survival.[26-28] More abundant expression of TUBB3, as observed in hMPV infected cells at 12 h suggests an initial mechanism for defense against oxidative stress which might eventually fail at 24 h after infection or points towards the blocking of cell cycle progression and induction of apoptosis.[26, 29, 30]
Less abundant expression of the other 4 proteins also points towards apoptotic processes in A549 cells at 24 h after infection. The molecular chaperones HSPA5 and HSP90AA1 are involved in the ER stress response upon accumulation

Table 5. Proteins involved in cell death

Spot nr.	protein ID	Name	RSV			hMPV			PIV			MV		
			6	12	24	6	12	24	6	12	24	6	12	24
323	P18206	VCL			-4.39/-2.54									
578	P35241	RDX	-/11.12		-/-2.14				2.82/-				3.82/-	
629	P20700	LMNB1						-4.22/-6.17						
841	Q13509	TUBB3					3.08/2.38							
522	P28331	NDUFS1												7.06/-
311	P19338	NCL						-2.65/-3.25						
566	Q8TCS8	PNPT1									10.85/13.72			
486	P13010	XRCC5	3.29/3.70											
525	P13010	XRCC5	2.52/2.44											
799	P54727	RAD23B				4.33/-								
425	P13639	EEF2						-2.62/-						
428	P13639	EEF2			-4.37/-4.24									
707	P07237	P4HB			-/-2.50	2.13/-			2.55/-					
311	P55072	VCP						-2.65/-3.25						
898	P30838	ALDH3A1												-/2.13
397	P07900	HSP90AA1					4.48/5.95							
519	P11142	HSPA8								-/5.83				
554	P11021	HSPA5						-2.23/-3.01						
631	P11142	HSPA8				-/2.21				-/-2.89				
719	P49368	CCT3									-2.38/-			
726	P49368	CCT3										-4.32/-		
737	P48643	CCT5						-2.15/-2.46						
767	P40227	CCT6A									-4.90/-2.91			
353	Q8WUM4	PDCD6IP			-3.42/-2.40			-3.29/-						-2.43/-
1791	Q15121	PEA15										-2.68/-		
1174	P06748	PNM1									4.90/3.31			

[a,b]Numbers indicate fold changes in protein expression upon infection with live (a) and UV-inactivated (b) virus particles compared to mock-infected cells.
Only significant changes are shown. - not significantly changed

of unfolded proteins in the ER.[31] When the unfolded protein response is not correctly activated, cells die by apoptosis.[32] In contrast to some other viruses, hMPV induces a less abundant expression of HSPA5 protein expression in A549 cells at 24 h after infection[33-36], suggesting that the ER stress response is not correctly induced thus resulting in the subsequent induction of apoptosis in these cells.

HSP90AA1 has a very important function in the folding of cell regulatory proteins and the refolding of stress-denatured polypeptides.[37-39] In hMPV infected A549 cells we observed an initial increase in HSP90AA1 expression at 12 h after infection which suggests a cellular response to hMPV-induced ER stress initiated by an increase of unfolded or misfolded proteins. Recently, an association between HSP90 protein complex and lamin A/C has been observed after oxidative stress.[40] Also in our hMPV and PIV infected A549 cells we observe an increase in HSP90AA1 as well as lamin A/C suggesting a damaging oxidative stress response in these cells. The HSP90AA1-associated protein lamin A/C has been shown to be involved in viral infections being required for repressing HSV replication and more abundant expression in hMPV and PIV infected A549 cells supports such a response upon infection.[41]

The other lamin that was found to be differentially expressed upon hMPV infection is lamin B1 although in contrast to lamin A/C it was less abundant upon infection. Lamin B1 also plays an important role in the cellular response to oxidative stress.[42] As a consequence of less abundant expression of lamin B1, hMPV infected cells might become more susceptible to oxidative stress and may result in subsequent cell death.

The molecular structure of P4HB is identical to that of the enzyme protein disulfide isomerase (PDI) which is known to protect cells against ER stress and inhibition of the protein will result in ER stress and subsequent induction of apoptosis.[43-45] P4HB is less abundant at 24 h after infection with RSV suggesting the induction of apoptosis in these cells. In hMPV and PIV infected cells this protein is more abundant at 6 h after infection suggesting the initiation of a protective response against ER stress.

VCP is involved in many cellular processes such as protein degradation and membrane fusion and has chaperone activity. Less abundant expression of VCP expression has been shown to cause ER and oxidative stress and thereby induces apoptosis through caspase activation while more abundant expression results in anti-apoptotic responses.[46] VCP shows less abundant expression at 24 h after infection with hMPV and suggests the induction of apoptosis.

Disruption of Cellular Structures

CCT3, CCT5, and CCT6 are all part of the T-complex protein 1 that is involved in the folding of actin and tubulin as well as many other newly synthesized proteins.[47, 48] Less abundant expression of CCT5 as observed in the hMPV, PIV and MV infected A549 cells has also been described for enterovirus 71 infected cells and a role in the disruption of the cytoskeletal structure to aid viral replication was suggested.[49] A supportive finding for this disruption of cytoskeletal structure is that the F-actin cross-linking protein ACTN4 expression is less abundant at 24 h after infection thus facilitating viral replication.[50] A similar result was observed for PDCD6IP, a protein that has been shown to play a role in infection with enveloped viruses, like human immunodeficiency virus type 1 (HIV-1).[51-54] In addition, PDCD6IP/AIP1 can associate with different cytoskeleton elements such as focal adhesion kinase and other cytoskeletal elements.[55] Less abundant expression of PDCD6IP as observed at 24 h after infection with RVS, hMPV and MV suggests the inhibition of cytoskeleton assembly.[56]

Vinculin (VCL) is a key regulator of focal adhesions that directly interacts with talin and actin and controls cell adhesion and migration. Interaction between VCL and the plasma membrane is essential for these processes.[57] Several viruses are known to disrupt or limit the function of these focal adhesions.[58-60] In glioblastoma cells, less abundant VCL expression is associated with induced apoptosis showing that disruption of the actin-VCL-cytoskeleton matrix is a major component of induced apoptosis in these cells.[61] Decreased expression of VCL by RSV infection in our experiment could suggest that the A549 cells have decreased adhesive and increased migratory capacities upon RSV infection, but might also suggest that apoptosis is induced in these cells.

Radixin (RDX) forms a complex with ezrin and moesin (ERM complex) that regulate membrane protein dynamisms and cytoskeleton rearrangements, are determinants in viral susceptibility, and are involved in apoptosis.[62-64] In RSV, PIV and MV infected A549 cells, expression of RDX was shown to be more abundant at 6 h after infection suggesting an initial defense response to the virus. At 24 h after RSV infection, however, RDX expression was less abundant suggesting increased apoptosis in RSV infected A549 cells.

Nucleolin (NCL) is an abundant nucleolar protein that has been implicated in chromatin structure, rDNA transcription, rRNA maturation, ribosome assembly and nucleo-cytoplasmic transport.[65] NCL is also involved in the stabilization of the apoptosis suppressor BCL-2. Reduced expression of NCL results in BCL-2 mRNA instability and decreased levels of BCL-2 protein resulting in increased

apoptosis.[66-68] NCL expression is less abundant upon infection with hMPV and thus also points towards induction of apoptosis in A549 cells.
Elongation factor 2 (EEF2) is a conserved monomeric GTPase involved in protein synthesis and translation elongation. This protein has been shown to have a role in HIV1 infection as a regulator of apoptosis and host innate cellular responses against viral factors as over expression of the protein induces protection against HIV1 induced apoptosis. In RSV and hMPV infected A549 cells, EEF2 is strongly less abundant at 24 h post-infection suggesting that induced apoptosis is no longer being suppressed by this protein.[69]
PEA15 is a 15-kDa phosphoprotein that can inhibit proliferation and that regulates the ability of BCL-2 to suppress Fas-induced apoptosis in a phosphorylation-dependent manner. In addition, PEA-15 phosphorylation is mediated by the PTEN/PI3K pathway.[70-71] PEA15 is less abundant in MV infected cells at 6 h after infection, which suggests that proliferation and BCL-2-mediated suppression of Fas-induced apoptosis is intact and proliferation is not suppressed yet.

CONCLUSIONS

The respiratory viruses RSV, hMPV, PIV and MV altered the expression of proteins involved in cell death that points towards an induction of apoptosis upon infection. Induction of apoptosis in epithelial cells by RSV and hMPV has been described before and our findings of induced ER stress, Bcl-2 and p53-dependent apoptosis support these data.[13-16] In addition, we newly identified proteins that are altered by the viral infection and induce apoptosis in A549 cells. For some of the identified proteins a role in virus-induced apoptosis has been previously described for viruses other than the respiratory viruses that were used in this experiment. However, several proteins have been newly identified, which are considered to be involved in respiratory virus infection-induced apoptosis. This list of proteins is likely to be further extended by improving the 2-D DIGE analysis, i.e. by increasing the number of replicates in the analysis or by using a different internal standard. This might allow the analysis of additional proteins and might also reliably allow the identification of spots with minor changes in protein expression (FC<2.5). In addition, complementary (semi-)quantitative proteomics-based techniques such as ICAT, ITRAQ or label-free MS analyses are also expected to confirm and are likely to complement our current findings. These proteins are of interest for further exploration of respiratory virus-induced apoptosis and might be interesting targets for the development of future therapeutics or prevention strategies against these severe disease causing viruses.

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Chapter 8

Host proteome correlates of vaccine-mediated enhanced disease in a mouse model of respiratory syncytial virus infection

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ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infections in infants. Despite over 50 years of research, to date no safe and efficacious RSV vaccine has been licensed. Many experimental vaccination strategies failed to induce balanced T-helper responses and were associated with adverse effects such as hypersensitivity and immunopathology upon challenge. In this study we explored the well-established recombinant vaccinia virus RSV F/G vaccination-challenge mouse model to study phenotypically distinct vaccine mediated host immune responses at the proteome level. Mass spectrometry-based spectral count comparisons enabled us to identify seven host proteins for which expression in lung tissue is associated with an aberrant T-helper 2 (Th2) skewed response characterized by the influx of eosinophils and neutrophils. These proteins are involved in processes related to the influx of eosinophils directly (Epx), chemotaxis and extravasion processes (Chil3) as well as eosinophil and neutrophil homing signals to the lung (Itgam). In addition, the increased protein levels of Arg1 and Chil3 point at a functional and regulatory role for alternatively activated macrophages and type 2 innate lymphoid cells in Th2 cytokine driven RSV vaccine-mediated enhanced disease.

IMPORTANCE

RSV alone is responsible for 80% of acute bronchiolitis cases in infants and substantial mortality in developing countries. Clinical trials with formalin-inactivated RSV vaccine preparations in the 1960s failed to induce protection upon natural RSV infection, and even predisposed for enhanced disease. Despite the clinical need, to date no safe and efficacious RSV vaccine has been licensed. Since RSV vaccines have a tendency to prime for unbalanced responses associated with an exuberant influx of inflammatory cells and enhanced disease, a detailed characterization of primed host responses has become a crucial element in RSV vaccine research. We investigated the lung proteome of mice challenged with RSV upon priming with vaccine preparations known to induce phenotypically distinct host responses. Seven host proteins have been identified which expression levels associated with vaccine mediated enhanced disease. The identified protein biomarkers support the development, as well as the detailed evaluation of next generation RSV vaccines.

INTRODUCTION

Respiratory syncytial virus (RSV) is the leading cause of severe lower respiratory tract infections (LRTIs) in children, being responsible for 80% of cases of acute bronchiolitis and subsequent hospital admission.[1,2] It has been estimated that RSV causes 33.8 million acute LRTIs globally each year, resulting in 66,000 – 199,000 deaths among children under 5 years of age, 99% of which occur in developing countries.[3] In addition to young children, immunocompromised individuals and the elderly are at increased risk for severe RSV disease and hospitalization. A licensed RSV vaccine is currently not available and the development of a vaccine against RSV infection has proven to be very difficult. Formalin-inactivated RSV (FI-RSV) vaccine trials in the 1960s failed to induce protection upon natural RSV infection, and even predisposed for enhanced disease resulting in two deaths and hospitalization of 80% of the vaccinated subjects.[4-7] Experimental inactivated- and subunit vaccines tend to prime for the induction of unbalanced, Th2 type, host responses that result in enhanced disease accompanied with an influx of inflammatory cells.[8] On the other hand, development of live attenuated RSV vaccines that are more likely to induce balanced host responses appear difficult to tune and these live vaccines tend either to be over- or under-attenuated.[9,10] Several RSV infection animal models have been developed and these are exploited for vaccine research and studies on virus induced (immuno)pathology. Multiple virological and immunological parameters are assessed in these studies to monitor the virus-host interaction. More recently "high-resolution" genomics tools have also been applied, in particular to characterize the host response. Previously, the FI-RSV mouse model has been used to characterize the host lung response in enhanced disease by mRNA profiling and several host gene expression correlates of enhanced disease have been identified.[11] To avoid the non antigen specific component at the basis of the host response and induced enhanced disease in the FI-RSV model[12] we extended this approach by exploring the recombinant vaccinia virus rVV-F/G vaccination-challenge model of enhanced disease. For this model it has been shown that vaccination of mice with rVV-G results in the production of neutralizing antibodies and subsequent reduction in RSV replication upon challenge infection.[13,14] However, these mice develop severe illness and pulmonary eosinophilia. Therefore, this model is ideally suited to study vaccine-enhanced disease upon RSV infection. As for rVV-G, priming with rVV-F results in the production of neutralizing antibodies but induces a more effective immunity against viral replication than rVV-G[13], no pulmonary eosinophilia is observed in rVV-F primed animals upon RSV challenge.

Using this mouse model of vaccine-enhanced disease and by applying mass spectrometry-assisted protein profiling we studied the molecular mechanism underlying vaccine induced RSV specific enhanced disease. With a comparative proteomics approach we identified seven proteins as biomarkers of enhanced disease. These proteins include Epx, Chil3 and Itgam, representing proteins specific for the infiltrating eosinophils. In addition, proteins that are postulated to play a role in the preceding stages of induction and establishment of skewed vaccine-induced hypersensitivity response, ArgI, Clca3, PurB, and Hk3, have also been identified. These seven protein biomarkers can be used to support the development, as well as the detailed evaluation, of next generation RSV vaccines.

MATERIALS AND METHODS

Cells and Viruses

HEp-2 and RK-13 cells were cultured in DMEM and RPMI1640, respectively (both BioWhittaker, Verviers, Belgium), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Greiner, Frickenhausen, Germany), penicillin (100 U/ml; BioWhittaker), streptomycin (100 µg/ml; BioWhittaker), L-glutamine (2 mM; BioWhittaker) and b-mercaptoethanol (10-5 M; Merck, Darmstadt, Germany) at 37°C and 5% CO2. RSV-A2 (a kind gift from P.J.M. Openshaw, National Heart and Lung Institute, Centre for Respiratory Infection, Imperial College London, London, UK) was propagated on HEp-2 cells. Stocks of the parental vaccinia virus (VV-wt) and recombinant vaccinia viruses expressing the RSV fusion protein (rVV-F) and attachment protein (rVV-G) were produced on RK-13 cells. Infectivity of all virus stocks was checked on the corresponding cell line by serial 10-fold dilutions and titers were calculated using the method of Reed and Muench.[15]

Mouse Vaccination and Challenge Studies

Six-to-eight weeks old female BALB/c mice (Netherlands Vaccine Institute, Bilthoven, The Netherlands) were vaccinated by scarification at the tail base with 106 PFU wt-VV, rVV-F or rVV-G virus as described before.[14, 16] Three weeks after vaccination, the mice were challenged by intranasal inoculation with 5x106 tissue culture infectious dose50 (TCID50) RSV-A2. Weight was measured on a daily basis and mice were sacrificed by exsanguination under isoflurane anesthesia at 1, 2, 3, 5, and 7 days after RSV challenge (10 animals per group

per day). Five animals from each group were used for broncho-alveolar lavage (BAL). Subsequently, lungs were inflated with 10% neutral-buffered formalin (formalin; Klinipath, Duiven, The Netherlands) and stored in 10% formalin for histopathological examination. The other five animals per group were used to dissect the lungs. Half of the lungs were transferred to virus transport medium for virus isolation, while the other half was stored in RNAlater (Ambion, Austin, USA) for RNA and protein isolation. To obtain control samples, naïve age-matched animals were sacrificed and processed identically. The study was approved by the Animal Ethics Committee, and was carried out in accordance with animal experimentation guidelines.

BAL Phenotyping

Cells in BAL were analyzed by flow cytometry as described before.[17] In short, BAL samples were centrifuged and residual red blood cells were lysed using red blood cell lysis buffer (Roche Diagnostics GmbH, Mannheim, Germany) for 20' at room temperature. Cells in BAL were counted and labeled with fluorescent monoclonal antibodies in phosphate buffered saline (PBS) supplemented with 3% HI-FBS for 60 minutes on ice. Next, cells were washed once with PBS and analyzed by fluorescence activated cell sorting (FACS).

Virus Isolation

Virus was isolated from lung tissue by homogenizing half of the lung in 1ml virus transport medium in the Fastprep-24 instrument (MPbio, Illkirch, France) using 1/4" ceramic spheres (VWR, Radnor, USA) according to the manufacturer's protocol (20' and 10 m/s). The homogenate was clarified by centrifugation and the supernatant was applied on a monolayer of HEp-2 cells in serial 2-fold dilutions. Cells were screened microscopically for cytopathic effect (cpe) and TCID50 was calculated as described above.

Protein Isolation

After removal of the RNAlater, the lungs were homogenized and lysed in Trizol and proteins were isolated from the interphase and organic phase that remained after RNA extraction from Trizol samples. Four volumes of ice-cold (-20˚C) acetone were added to these fractions and incubated at -20˚C for 1 hour. Precipitated proteins were then centrifuged at maximum speed for 5 minutes.

The protein pellet was washed twice with ice-cold 80% acetone. The pellet was air-dried and suspended in 40µl lysis buffer (30mM Tris, 7M urea, 1M thiourea, and 4% CHAPS). Protein concentration in each sample was determined using the 2-D Quant kit (GE Healthcare, Amersham, UK) according to the manufacturer's instructions.

Mass Spectrometry

A total amount of 25mg protein was loaded onto a 12% SDS-PAGE gel and run at 100V until the front reached the bottom of the gel. The gel was stained overnight with Coomassie Blue Silver. Each lane was divided into 5 slices and each slice was cut into smaller pieces and prepared for protein digestion with trypsin. Peptides were extracted using 2% trifluoroacetic acid (TFA) and buffer B (80% acetonitrile (ACN), 0.5% acetic acid, 1% TFA), and subsequently desalted and concentrated using C18 StageTips and buffer A (0.5% acetic acid, 1% TFA). Peptide mixtures were purified and desalted using C18-stage tips. Peptide separation and sequence determination was performed with a nano-high performance liquid chromatography system (Agilent 1100 series, Amstelveen, the Netherlands) connected to a 7-T linear quadrupole ion trap-ion cyclotron resonance Fourier transform mass spectrometer (Thermo Electron, Breda, the Netherlands). Peptides were separated on a 15-cm 100-µm-inner-diameter PicoTip emitter for online electrospray (New Objective, Woburn, MA) packed with 3µm C18 beads (Reprosil, Dr Maisch GmbH, Ammerbuch-Entringen, Germany) with a 60-minute linear gradient from 2.4 to 40 percent acetonitrile in 0.5% acetic acid at a 300nl/min flow rate. The four most abundant ions were sequentially isolated and fragmented in the linear ion trap by applying collisionally induced dissociation. Proteins were identified using the MASCOT search engine (Matrix science, London, UK) against the human International Protein Index (IPI) database (forward and reverse sequences) using the following search criteria: 20 ppm peptide tolerance, a maximum of 2 missed cleavages, a fixed carbamidomethyl modification of cysteines, and variable oxidation (M) and deamidation (NQ) modification. UniProt KB mapping table was used to map IPI IDs to UniProt identifiers (ftp://ftp.uniprot.org/pub/databases/uniprot/current_release/knowledgebase/idmapping/). Differentially expressed proteins were functionally classified using GO-slim identifiers[18] and Ingenuity Pathway Analysis (Qiagen, Redwood City, USA).

Validation of Peptides and Proteins

Mascot database search files were further processed using MSQuant 1.4.3 to generate first ranked peptide lists. A validation filter was applied to these lists using the reverse database searches. First the mass accuracy cut-off was set at the mass relative error (parts per million (ppm)) at which 95% of all identified peptides were still included. Then the reverse database search was used to set the peptide cut-off score at which only 10% of the identified peptides were still included in the reverse database search list. Then the number of peptide sequences per protein was calculated and the list was further validated by the following criteria; proteins that were identified based on 3 peptides or more were accepted. If 2 peptides were identified, these peptides were not allowed to contain any modifications. If only 1 peptide was identified, no modifications were accepted, peptide scores needed to be above 40 and the peptide delta score should be above 10. This resulted in false positive rates of 11% for proteins identified with 1 or 2 peptides and 0.9% for proteins identified with 3 or more peptides. After internal calibration of peptides masses by MSQuant and validation as just described, the absolute mass accuracy of all identified peptides was below 15ppm for all samples with an average of ~2ppm. To remove redundancy at the protein level and to uniquely assign peptides to one protein, the peptides were remapped using Protein Coverage Summarizer (http://ncrr.pnl.gov/software/).

Comparative Protein Expression Analyses

For each protein in the validated list of proteins, the number of unique parent ions (spectral count) was calculated based on peptide sequences and modifications. The number of spectra matched to peptides from a protein is used as a surrogate measure of protein abundance.[19] A negative binomial model that handles overdispersion caused by few biological replicates is used to estimate the per-protein counts.[20,21] To test for differential expression between two conditions, the mean of the normalized per-protein counts in each condition is computed and compared for equality. An exact type p-value defined as the probability of a pair of observed counts is computed as previously described.[22] After computing the normalized mean counts in each condition and the corresponding p-values, we controlled for multiple testing by controlling the false discovery rate (FDR) defined as expected proportion of false rejection among the rejected hypotheses, using the Benjamini and Hochberg (BH) procedure.

RESULTS

Induction of Skewed Immune Responses in an RSV Vaccination-Challenge Mouse Model

BALB/c mice were primed with rVV-F, rVV-G, or VV-wt and challenged with RSV. Bodyweight, viral titers in the lungs, cellular composition of BAL samples were measured, and histopathological examination of the lungs was performed for a period of seven days. Bodyweight measurements showed that VV-wt primed animals remained at starting weight upon challenge with RSV (Figure 1A). In contrast, animals that have been primed with the RSV fusion protein (rVV-F) or the RSV attachment protein (rVV-G) showed a reduction in body weight to approximately 85% of starting weight in 4 to 5 days. Subsequently, the rVV-F primed animals returned to starting weight whereas the rVV-G primed animals remained low at 85% of starting weight until the end of the study at day 7 (Figure 1A).

Virus could be isolated from lung homogenates from all animals in the VV-wt group. Virus titers from VV-wt primed mice that mount a primary immune response to RSV peaked at day 3 to a level of 106 TCID50/gram lung tissue. At any time point, the highest titers obtained in rVV-F or rVV-G primed mice were at least 10-fold lower than those obtained for the VV-wt primed mice. In addition, the virus titers in these groups of mice showed an earlier and much lower peak (day 2) than VV-wt primed mice, pointing at partial protection from challenge in rVV-F and rVV-G primed mice (Figure 1B).

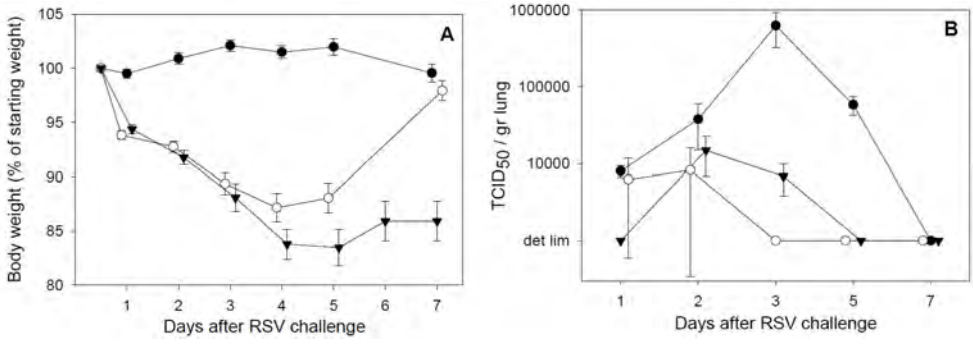

Figure 1. Relative body weight (panel A) and replication competent virus titers in the lung (panel B) from VV-wt (black dots), rVV-F (white dots), and rVV-G (black triangles) vaccinated mice up to seven days following RSV challenge. Average numbers with standard error are indicated.

BAL samples were obtained at day 1, 2, 3, 5, and 7 after challenge and the cellular composition was analysed by FACS analysis. Mice primed with VV-wt displayed a typical primary response to RSV infection with a relatively late and modest influx of cells during the experiment. Mice primed with rVV-F displayed the highest numbers of T-cells, B-cells, and dendritic cells in the BAL samples, while neutrophils and eosinophils were more abundant in BAL samples obtained from rVV-G primed animals (Figure 2). In fact eosinophil numbers in BAL samples contrasted most between rVV-F and rVV-G mediated secondary responses: high numbers were found for rVV-G primed animals while eosinophils were virtually absent in BAL samples from rVV-F primed animals (Figure 2D).

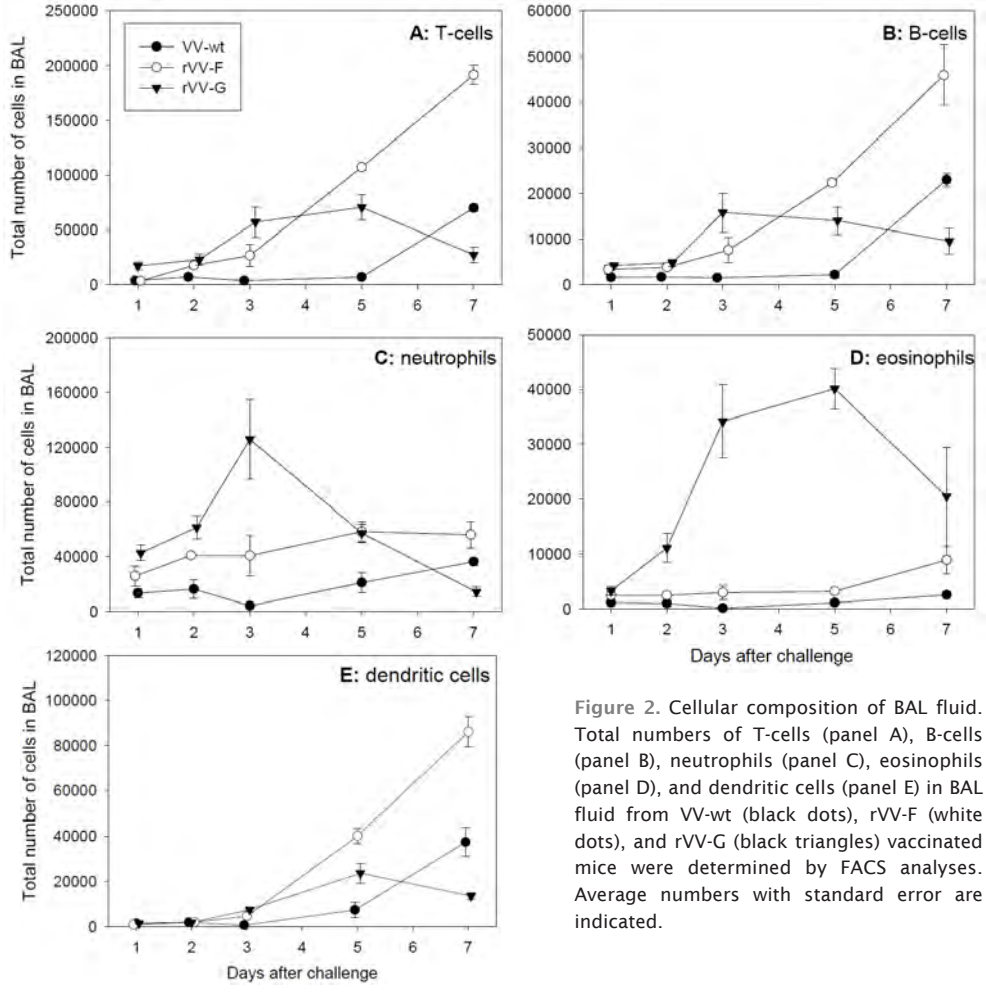

Figure 2. Cellular composition of BAL fluid. Total numbers of T-cells (panel A), B-cells (panel B), neutrophils (panel C), eosinophils (panel D), and dendritic cells (panel E) in BAL fluid from VV-wt (black dots), rVV-F (white dots), and rVV-G (black triangles) vaccinated mice were determined by FACS analyses. Average numbers with standard error are indicated.

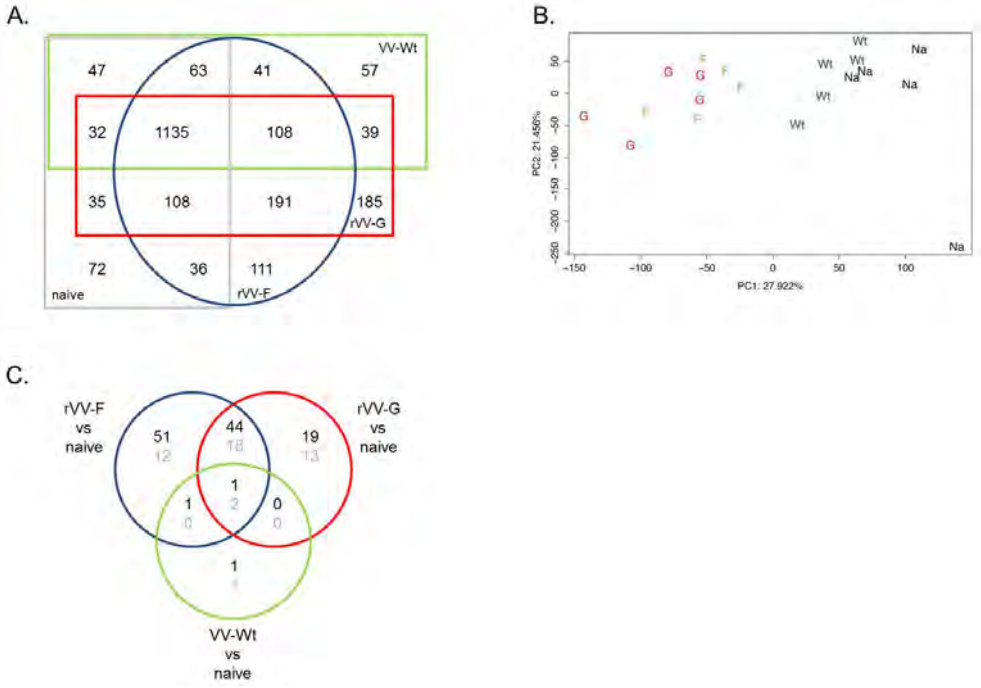

Figure 3. Proteome analysis of lung samples. Distribution of the number of proteins identified in each group of vaccinated mice upon RSV challenge (panel A). PCA analysis of all lung proteome samples on the basis of normalized spectral counts (panel B). The number of identified and validated proteins, up-regulated (black numbers) or down-regulated (grey numbers) upon RSV challenge in response to VV-wt, rVV-F or rVV-G vaccination compared to naive mice (panel C).

Histopathological examination of lung slides obtained from animals that were sacrificed at day 5 after challenge revealed that both VV-wt and rVV-F primed mice experienced interstitial pneumonia characterized by marked peribronchiolar and perivascular infiltrates. Conversely, rVV-G primed animals experienced a more severe broncho-interstitial pneumonia, in some animals even leading to foci of necrosis associated with the presence of viral antigen. Interestingly, rVV-G primed animals accounted for the highest positive staining of viral antigen of all groups. While eosinophils were virtually absent in VV-wt and rVV-F primed animals, these cells were readily detectable in rVV-G primed animals. Most eosinophils were located in the perivascular and peribronchiolar infiltrates and to a lesser degree in the alveolar walls and lumina (data not shown).

In line with the original observations obtained with this RSV vaccination and challenge model[13, 14] the data collectively demonstrate that the rVV-F and rVV-G priming regimes indeed mediate the induction of distinct host response phenotypes upon RSV challenge. The collected lung samples are a good substrate

to monitor protein expression profiles and to search for differences between the different priming regimes.

Mass Spectrometry-Assisted Protein Profiling of Phenotypically Distinct Host Responses

Lungs collected 5 days after RSV challenge, were homogenized, and proteins were isolated and subjected to FT-ICR-MS/MS analysis. The collected sample set was complemented with identical lung samples obtained from naïve mice. Raw FT-ICR-MS/MS data were processed and validated. Between 2,247 and 2,547 distinct proteins were detected for each group of mice. When comparing the different experimental groups of mice, a large overlap in detected proteins was observed (Figure 3A). Principal component analysis (PCA) on the normalized protein expression data (spectral counts) revealed two major clusters. One cluster consists of VV-wt primed and naïve mice derived lung samples while the other consists of rVV-F and rVV-G lung samples, indicating that protein expression in the two latter groups is similar but different from naïve and VV-wt primed mice (Figure 3B).

Next, we applied spectral count analysis to identify differentially expressed proteins between the vaccination groups and naïve mice. First, spectral counts between VV-wt, rVV-F and rVV-G vaccinated mice and naïve control mice were compared. Lungs from VV-wt vaccinated mice displayed least changes in protein expression with only 3 down- and 3 up-regulated proteins compared to naive mice (Figure 3C).

Table 1. Differentially expressed proteins in rVV-G vs rVV-F primed mice upon challenge with RSV

UniProt ID	gene	Protein name	rVV-G	rVV-F	rVV-G vs rVV-F	
			spectral count	spectral count	fold change	adjusted p-value
Q3U1U4	Itgam	Integrin alpha-M	3.8	0	up	0.002
P49290	Epx	Eosinophil peroxidase	12.2	1.0	12.1	0
Q61176	Arg1	Arginase 1	18.2	3.1	6.0	0.002
Q9D7Z6	Clca3	Calcium-activated chloride channel regulator 1	6.9	0	up	0
O35744	Chi3l3	Chitinase-like protein 3	5.7	0	up	0
Q3TRM8	Hk3	Hexokinase 3	2.8	0	up	0.041
O35295	Purb	Transcriptional activator protein Pur-beta	0	3.5	down	0.024

Vaccination with rVV-F or rVV-G induced higher numbers and more pronounced changes in protein expression (129 and 97 differentially expressed proteins, respectively). The magnitude of these total lung proteome changes reflects a relatively slow developing primary immune response (VV-wt primed animals) and a much faster developing secondary response in the RSV specific primed mice (rVV-F and rVV-G). Only 3 proteins were differentially expressed in all 3 vaccination groups (Figure 3C). As expected based on the PCA data, a large overlap was observed between the rVV-F and rVV-G vaccinated mice where 50%-67% of the identified differentially expressed proteins were also found in the other group. The rVV-F and rVV-G primed responses are indeed highly similar: only 7 proteins (Epx, Chil3, Itgam, Arg1, Clca3, PurB and Hk3) are significantly differentially regulated in a direct comparison of rVV-F and rVV-G primed lung tissue samples (Table 1). Six of these proteins are more abundantly present in lung tissue of rVV-G primed mice: 2 proteins are up-regulated (Arg1 and Epx) and 4 are unique for the rVV-G primed samples (Itgam, Chi3l3, Clca3, and Hk3). One protein (Purb) was expressed in rVV-F primed mice only.

rVV priming and RSV challenge RSV challenge after VV-wt priming leads to only a few differentially expressed proteins in lung tissue at day 5: Eif2s1, Gpd1l, and Armc10 are up-regulated compared to naïve mice while Msra, Hba-a1/2 and

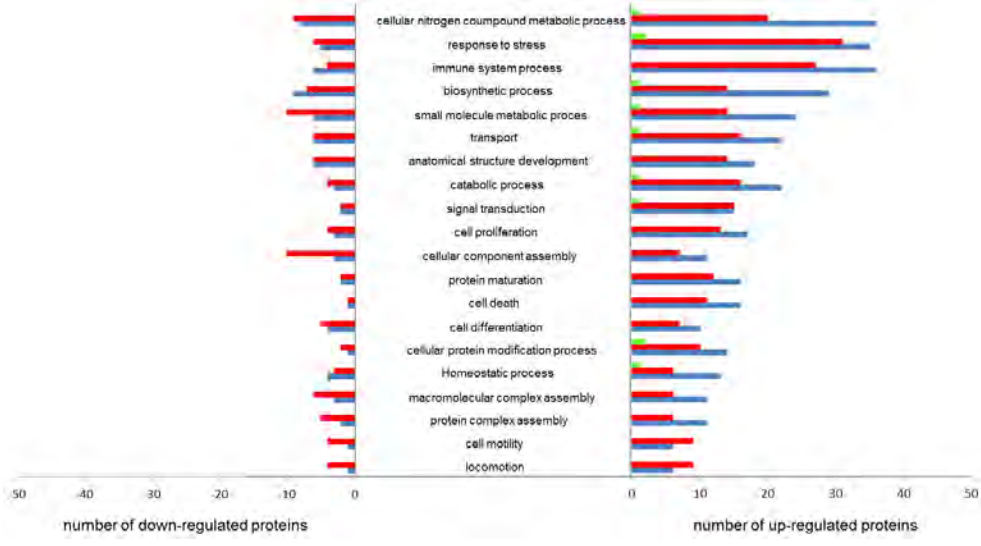

Figure 4. Functional classification of differentially expressed proteins. Proteins were grouped by functional category derived from GO slim and UniProt (http://www.uniprot.org/uniprot/). The graph shows the absolute number of differentially expressed proteins in VV-wt (green bars), rVV-F (blue bars), and rVV-G (red bars) vaccinated mice upon RSV challenge compared to naïve mice.

Krt34 are down-regulated. These proteins are involved in responses to stress and metabolic processes (Figure 4). PCA analysis also showed that protein expression in lungs from VV-wt and naïve mice is highly similar and explains why only 6 differentially expressed proteins were observed (Figure 3C).
There were many similarities in the alteration of protein expression between rVV-F and rVV-G primed animals (Figure 3). A total number of 65 proteins were differentially expressed in both groups of mice compared to naïve mice and these mainly included proteins involved in metabolic processes and response to stress (Figure 4). A total of 63 proteins were differentially expressed in rVV-F only and 32 in rVV-G vaccinated mice only. In both groups, the majority of differentially expressed proteins were up-regulated involving similar numbers and biological processes (Figures 3 and 4).

Table 2. Differentially expressed proteins involved in immune system processes

UniProt ID	gene	Protein name	rVV-F vs naive		rVV-G vs naive		rVV-G vs rVV-F	
			FC	adj. p-value	FC	adj. p-value	FC	adj. p-value
P04187	Gzmb	Granzyme B	up	0.000	up	0.033		
Q02357	Ank1	Ankyrin-1	-5.2	0.000	-5.9	0.015		
P15508	Sptb	Spectrin beta chain, erythrocytic	-6.2	0.000	-30.7	0.011		
Q8R2Q8	Bst2	Bone marrow stromal antigen 2	11.7	0.000	11.2	0.004		
Q9R233	Tapbp	Tapasin	up	0.000	up	0.002		
P08032	Spta1	Spectrin apha chain, erythrocytic 1	-7.1	0.000	-10.6	0.002		
P36371	Tap2	Antigen peptide transporter 2	up	0.001	Up	0.001		
Q64282	Ifit1	Interferon-induced protein with tetratricopeptide repeats 1	up	0.001	up	0.001		
Q60766	Irgm1	Immunity-related GTPase family M protein 1	up	0.000	up	0.000		
Q5I2A0	Serpina3g	Serine proteasse inhibitor A3G	up	0.000	up	0.000		
Q64345	Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	up	0.000	up	0.000		
Q9QZ85	Iigp1	Interferon-inducible GTPase 1	130.7	0.000	98.4	0.000		
Q01514	Gbp1	Interferon-induced guanylate-binding protein 1	52.3	0.000	44.2	0.000		
O35955	Psmb10	Proteasome subunit beta type-10	35.1	0.000	37.2	0.000		
P42225	Stat1	Signal transducer and activator of transcription 1	30.0	0.000	30.0	0.000		
P21958	Tap1	Antigen peptide transporter 1	up	0.000				
P01029	C4b	Complement C4-B	38.4	0.000				
P01863	Ighg	Ig gamma-2A chain C region, A allele	8.6	0.000				
P29351	Ptpn6	Tyrosine-protein phosphatase non-receptor type 6	up	0.001	up	0.000		

Table 2. (*continued*)

UniProt ID	gene	Protein name	rVV-F vs naive		rVV-G vs naive		rVV-G vs rVV-F	
			FC	adj. p-value	FC	adj. p-value	FC	adj. p-value
P11835	Itgb2	Integrin beta-2	up	0.001	up	0.000		
P47941	Crkl	Crk-like protein	up	0.001				
Q61646	Hp	Haptoglobin	4.4	0.001				
Q9Z0E6	Gbp2	Interferon-induced guanylate-binding protein 2	10.0	0.002	8.3	0.038		
Q62418	Dbnl	Drebrin-like protein	28.5	0.002				
Q06318	Scgb1a1	Uteroglobin	-23.0	0.002				
Q61107	Gbp4	Guanylate-binding protein 4	up	0.004				
Q8CFB4	Gbp5	Guanylate-binding protein 5	up	0.006	up	0.001		
P97290	Serping1	Plasma protease C1 inhibitor	9.1	0.006				
Q64112	Ifit2	Interferon-induced protein with tetratricopeptide repeats 2	up	0.007				
O89053	Coro1a	Coronin-1A	2.8	0.012	3.2	0.012		
P02089	Hbb-b2	Hemoglobin subunit beta-2	-2.0	0.018				
P10711	Tcea1	Transcription elongation factor A protein 1	up	0.020				
P04441	Cd74	H-2 class II histocompatibility antigen gamma chain	up	0.022	up	0.007		
Q9EPB4	Pycard	Apoptosis-associated speck-like protein containing a CARD	10.2	0.023	13.5	0.005		
P31230	Aimp1	Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	16.4	0.023				
P00493	Hprt	Hypoxanthine-guanine phosphoribosyltransferase	3.0	0.024				
Q60710	Samhd1	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	2.2	0.027	2.3	0.030		
P18468	H2-Eb1	H-2 class II histocompatibility antigen, I-A beta chain	up	0.028	up	0.018		
Q99JB2	Stoml2	Stomatin-like protein 2, mitochondrial	up	0.028				
P01027	C3	Complement C3	2.0	0.042				
Q64339	Isg15	Ubiquitin-like protein ISG15	up	0.045				
P09470	Ace	Angiotensin-converting enzyme	-1.8	0.048	-3.9	0.002		
Q9EQH2	Erap1	Endoplasmic reticulum aminopeptidase 1			15.7	0.044		
Q61233	Lcp1	Plastin-2			2.1	0.040		
P16110	Lgals3	Galectin-3			3.5	0.037		
P01915	H2-Eb1	H-2 class II histocompatibility antigen, E-D beta chain			17.5	0.032		
P49290	Epx	Eosinophil peroxidase			34.0	0.007	12.0	0.000
Q3U1U4	Itgam	Integrin alpha-M			up	0.001	up	0.000

Proteins are known to be involved in multiple pathways, and hence, are likely to have multiple functions. Pathway analysis (Ingenuity) was performed for further functional classification of differentially expressed proteins, and to investigate the presumed interactions between these proteins within specific functional categories. A substantial proportion of the differentially expressed proteins in rVV-F and rVV-G primed mice appeared to play a role in antigen presentation (7.2%), biosynthesis (6.6%) or metabolism (7.8%), but the majority is involved in immune and defence responses (16.3%). Within the latter group of differentially expressed proteins five (Npm1, Snd1, Kars, Krt18, and Eif4a1) have been reported to be involved in host-virus interactions and another seven (Bst1, Bst2, Gbp3, Ifit1, Ifit2, Ifit3, Ddx21, and Gbp1) have shown to be active in antiviral immune responses. Within this group of proteins involved in antiviral immune responses Gbp3, Ifit2, and Ddx21 were found to be up-regulated compared to naïve mice in rVV-F primed mice only, while Bst2, Ifit1, Ifit3 and Gbp1 were up-regulated in both rVV-F and rVV-G primed mice. A first line of defense against virus infections is formed by the type I interferon induced proteins. Five proteins in the list of differentially expressed proteins were involved in this defense response. Ifit1, Ifit2, and Ifit3 are members of the interferon-induced protein with tetratricopeptide repeats (IFITs) family and Gbp1 and Gbp3 are members of the IFN-induced guanylate-binding proteins which are all involved in anti-viral responses.[23, 24] Ifit1 and Ifit3 were differentially expressed in rVV-F and rVV-G primed mice while Ifit2 was only found differentially expressed in rVV-F primed mice. All three proteins are expressed in the cytoplasm and mitochondria but Ifit2 is expressed in microtubules as well and interacts with the cytoskeleton that may explain the difference observed.

DISCUSSION

Vaccines aim to induce antigen specific memory responses that mediate safe and effective secondary responses when the host is exposed to the pathogen. Many live-attenuated and inactivated virus vaccines have been developed and have collectively reduced the incidence of disease. However, for RSV and several other viruses, classical formalin-inactivated vaccine production approaches failed as they prime for unbalanced, skewed Th2 type immune responses upon challenge.[7, 25, 26] These hypersensitivity responses are associated with immune pathology and are marked by peribronchiolar monocytic infiltration including neutrophils and eosinophils.[27] In this study we explored the well-established

rVV-RSV vaccination-challenge mouse model to identify protein markers and regulatory mechanisms that are associated with or are underlying vaccine induced aberrant host responses. When comparing lung tissue samples from rVV-F and rVV-G primed animals with control samples from naïve mice we identified many differentially regulated proteins. These proteins are involved in metabolic processes and stress, but a significant part was identified as host immune response related proteins (Table 2). By directly comparing vaccine induced host responses, we were able to uncover subtle but highly relevant lung proteome differences between phenotypically distinct Th1- and Th2-like host responses. Direct comparison of protein expression in lung tissue from RSV challenged mice upon vaccination with rVV-F and rVV-G resulted in differential expression of 7 proteins only: Epx, Chil3, Itgam, Arg1, Clca3, PurB and Hk3. These proteins are of particular interest as their biological functions can be linked to the vaccination-induced host response phenotype and immunopathology.

An important feature of RSV vaccine primed Th2 skewed host responses is a marked increase of eosinophils into the lungs. As expected, rVV-G primed mice indeed accumulated significant numbers of eosinophils in the lungs (BAL) upon RSV challenge (Figure 2). Eosinophil peroxidase (Epx) is expressed by eosinophils and is localized in the cytoplasmic granules. Against a background of many other cells present in lung tissue samples, we could easily detect increased levels of the Epx protein in the lungs of rVV-G primed animals (Table 1). Upon RSV challenge, the Epx protein was present at much lower levels (12 fold) in the lungs of rVV-F primed animals. The Epx protein functions as an oxidant that has been shown to be released at sites of infection to mediate lysis of protozoa or parasitic worms. Lung histology from the two fatal cases of the FI-RSV trial in the sixties revealed a pronounced inflammatory lung infiltrate including eosinophils and neutrophils.[7, 28] This result has subsequently been reproduced in RSV vaccination-challenge animal models.[29] Especially the association of lung eosinophilia with enhanced disease has attracted much attention but is most likely a secondary event; rVV-G primed eosinophil deficient mice also display enhanced disease upon challenge with RSV.[30] Nevertheless, vaccine-primed eosinophilia reflects an unbalanced host response to be avoided as influx of these cells is regulated by Th2 type cytokines including IL-4, Il-10, and IL-13.[31] In the context of RSV infection, the Epx protein can be used for vaccine evaluation purposes. Increased Epx mRNA levels have not been observed in rodent RSV vaccination-challenge models of enhanced disease.[11] (unpublished observation).

The Chil3 protein levels are also up-regulated in rVV-G-primed animals relative to those primed with the F protein. Chil3, chitinase-like-protein 3, is a secretory

protein (also known as Ym1) with in vitro chemotactic activity for T-lymphocytes, bone marrow polymorphonuclear leukocytes and eosinophils. In addition, it has been demonstrated that Chil3 specifically triggers extravasion of eosinophils. The Chil3 protein shares conserved amino acid residues with the chitinase family of proteins, but also harbors a conserved CXC chemokine motif near the NH2 terminus.[32] This hybrid type protein is mainly produced by macrophages and is localized in the lumen of the rough endoplasmic reticulum and the nuclear envelope of these cells and of neutrophils.[33] In a murine model of airway hyper responsiveness (AHR) Chil3 mRNA was also up-regulated in the lungs. Interestingly, up-regulation preceded the onset of airway inflammation in this model and specific inhibition of Chil3 mRNA expression suppressed the induction of eosinophilia supporting a critical role for Chil3 in eosinophilic inflammation processes.[34] Up-regulation of Chil3 protein in the rVV-G primed mice upon RSV challenge suggests a role for Chil3 in the observed eosinophilia in these mice.

Homing of effector cells in inflamed tissue is facilitated by many cell adhesion factors. In line with the pronounced influx of different cell types into the lungs of rVV-F and rVV-G primed animals upon challenge with RSV, we identified Itgam, Itgb2, Lgals3bp, Lama5 and Tns1 as being differentially expressed relative to naïve animals. However, only the Itgam protein expression level was significantly increased in lung tissue obtained from rVV-G vaccinated mice when comparing rVV-G- and rVV-F-primed animals directly. Itgam appeared to be induced in rVV-G-primed and not in rVV-F-primed mice. The Itgam gene encodes for the CD11b-integrin (alphaM) polypeptide, which pairs with the polypeptide integrin beta-2 (Itgb2 = CD18-integrin) to form a functional integrin also known as the complement receptor type 3 (CR3) or macrophage-1 antigen (Mac-I). Itgb2 was also up-regulated in rVV-G primed mice and indicates an increased expression of the functional integrin. In contrast to Itgam, which was only detected in rVV-G primed mice, Itgb2 was also found to be up-regulated in rVV-F primed mice compared to naïve mice and with no statistical difference in expression compared to rVV-G primed mice (Table 2). Indeed, Itgam binds to multiple ligands and is involved in other biological processes in addition to cell adhesion.[35] Itgam expression was originally reported to be specific for neutrophils and monocytes/macrophages. However, Itgam expression was also induced by IL-5 and IL-33 in eosinophils.[36] In a rVV-G-primed Th2 skewed host response, Itgam expression can thus mediate the recruitment of neutrophils as well as eosinophils to the lungs. The concerted action of up-regulated Chil3 and Itgam proteins may account for the high influx level of eosinophils in the lungs of the rVV-G-primed animals.

Arginase-I (ArgI) catalyzes the hydrolysis of arginine to ornithine and urea, and is constitutively expressed in hepatocytes[37] but has also been recognized as an enzyme involved in the iNOS pathway in myeloid cells that is predominantly regulated by exogenous stimuli.[37, 38] In cells isolated from human blood, Arg1 expression has so far only been found in neutrophils.[39] In mice, Arg1 expression has been reported for alternatively activated macrophages (AAM). AAM play an important role in Th2 driven pathological conditions like asthma and express Arg1 together with Chil3, suggesting that these macrophages help to orchestrate the Th2 skewed responses in rVV-G-primed enhanced disease.[40] In addition, the elevated Arg1 levels in lungs from rVV-G-primed compared to rVV-F-primed mice may also point at a role for type 2 innate lymphocytes since these cells constitutively express Arg1.[41] Participation of the latter cell type may also be dependent on IL-33. This cytokine was not detected in our proteome screen. However, microarray analysis of lung tissue samples obtained from the same experiment showed that IL-33 mRNA level was increased for rVV-G-primed samples when compared with naïve lung samples. In a direct comparison between rVV-G- and rVV-F-primed lung tissue samples, the rVV-G-primed IL-33 lung mRNA signal was higher but did not reach statistical significance (data not shown).

Members of the chloride channels, calcium-activated (CLCA) family of proteins including the murine Clca3 (mClca3, alias gob-5) and its human ortholog hCLCA1 have been identified as relevant molecules in diseases with secretory dysfunctions, including asthma and cystic fibrosis.[42] mClca3 is also up-regulated in rVV-G-primed animals relative to rVV-F-primed animals. Biochemical analysis of the posttranslational processing and intracellular trafficking of the mClca3 protein showed that it does not form an anion channel as suggested by the protein name, but is cleaved in two sub-units that are fully secreted as a glycosylated protein complex into the extracellular environment.[42] Interestingly, it has been demonstrated that hCLCA1 mRNA levels and protein expression are significantly increased in the airway epithelium of asthmatic patients[43, 44], and overexpression of mClca3 in mice resulted in goblet cell metaplasia and mucin overproduction.[45] Targeting the mClca3 protein with antibodies inhibited these processes in asthmatic mice.[46] Collectively, the data suggest that CLCA proteins may also play an important role in the Th2 driven RSV vaccine induced enhanced lung disease and that detection of Clca3 protein expression could be used to guide RSV vaccine design and evaluation.

Hexokinase III (Hk3) and purine-rich element-binding protein Beta (PurB) both do not have an obvious function in the host response to infection. Hexokinases

catalyze the first step in glucose metabolism. Hk3 expression is relatively low in most tissues, with the highest levels reported in lung, kidney, and liver. Hk3 may play a role in protecting against cell death, and its overexpression decreases the oxidant induced production of reactive oxygen species (ROS).[47] In addition to a protein expression level that changes due to the influx of specific immune cells, the metabolic function of Hk3 can only be indirectly related to the observed host responses. PurB is the only protein that is expressed at lower levels in rVV-G-primed animals relative to rVV-F-primed animals. PurB is a sequence-specific, single-stranded DNA-binding protein implicated in the control of both DNA replication and transcription. Defects in this gene are associated with the development of acute myelogenous leukemia in which some hematopoietic precursors are arrested in an early stage.[48] A non-equal role for PurB in cell division for the distinct immune cell types present in the RSV challenged lungs may contribute to the skewed cell composition as observed for the different vaccine priming regimes.

In summary, by proteome analysis we have identified a set of host proteins that are differentially expressed between two phenotypically distinct secondary immune responses each evoked upon challenge with RSV after priming with closely related, but distinct, RSV vaccine preparations: rVV-F and rVV-G. Vaccination of mice with rVV-G- but not rVV-F- prior to RSV challenge induces typical vaccine mediated enhanced disease that is characterized by a marked influx of eosinophils and neutrophils. The identified differentially expressed host proteins between the two vaccination dependent conditions are involved in processes related to the influx of eosinophils directly (Epx), chemotaxis and extravasion (Chil3) as well as homing to the lungs of these cells and neutrophils (Itgam). In addition, the increased protein levels of Arg1 and Chil3 point at a functional and regulatory role for alternatively activated macrophages and type 2 innate lymphoid cells in Th2 cytokine driven RSV vaccine mediated enhanced disease. The identified proteins support research on regulatory aspects of vaccine induced skewed host responses, as well as the evaluation of next generation RSV vaccines.

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Chapter 9

General discussion

GENERAL DISCUSSION

One third of children hospitalized with bronchiolitis do not require supportive interventions and 4.6-6.8% of the patients initially discharged home, require hospitalization later on during infection due to deterioration.[1-3] One may therefore conclude that the predictive value of the currently available clinical assessment tools for severity of bronchiolitis is limited. Clinical prediction rules to predict safe discharge, length of hospitalization and ICU admission for children with acute viral lower respiratory tract infections (LRTIs) are based on demographic criteria including age, birth weight, having older siblings, and clinical symptoms such as respiratory and heart rate, oxygen saturation and duration of symptoms.[4-11] Although these prediction rules have the potential to improve clinical judgment, validation in other cohorts is often lacking and implementation in daily practice is difficult due to subjective criteria. A more objective and reproducible prediction of disease severity may be achieved by the use of additional biochemical and hematological markers.

In this thesis we evaluated the role of viral factors and host immune responses that contribute to disease severity in young children with viral LRTIs, especially respiratory syncytial virus (RSV) infections, and aimed to identify potential biomarkers to assess disease severity in young children with these infections.
We performed a prospective study in two hospitals in which 154 children under the age of 5 years with viral LRTIs were included during three consecutive winter seasons. Clinical data, blood samples and nasopharyngeal aspirates were obtained during acute infection and at recovery from previously hospitalized children. One of the strengths of this approach was that all patients were included by a single investigator, thus resulting in a low percentage of parental refusals, few missing clinical data and uniformly collected samples. This “respiratory tract infection biobank” was used for several studies in this thesis and follow-up studies are ongoing. In addition to the clinical study we also performed in vitro work using proteomic and transcriptomic technologies to assess the immune response of infected respiratory epithelial cell lines, and employed an experimental murine model to obtain insight in the pathogenesis of RSV infection and to identify leads for biomarkers, new treatment strategies and vaccine development.

The most important findings of the studies presented in this thesis are:

- Disease severity in young children with viral bronchiolitis is not associated with infection caused by multiple viruses or viral load

- Several inflammatory markers measured at the transcriptional level (MMP-9 and MMP-8) and in plasma (combination of IL-8, CCL-5 and CD4+ T-cell counts) discriminate severe from mild RSV infection

- Transcriptome analyses of patient material resulted in the identification of novel biomarkers for disease severity such as olfactomedin-4

- Proteome analyses of material obtained from in vitro and in vivo (mice) experimental models revealed several host proteins which are involved in innate immune responses

I VIRAL FACTORS

Multiple Infections

The use of molecular detection techniques has facilitated the simultaneous detection of pathogens in respiratory specimens. The prevalence of co-infections ranges between 19 to 35% in young children with viral respiratory tract infection admitted to hospital or evaluated at the emergency department.[12-14] Although many studies evaluated the association between infection with multiple viral pathogens and disease severity, the clinical impact of multiple virus infections on clinical presentation and outcome remains unclear.[13,15-17] The interpretation of results from these studies is complicated by differences in study design including the methods of pathogen detection, the types and copy number of viruses tested, the specific patient population being studied and other confounding factors. In the study presented in this thesis no association between disease severity and detection of more than one viral pathogen was observed. Even more remarkable, multiple infections in children older than 3 months had a less severe course of disease compared to infections with one viral pathogen. These results were confirmed in a new cohort of patients (I. Ahout et al, submitted).
In agreement with our findings, a less severe course of disease in children with bronchiolitis simultaneously infected by more viruses as compared to children

infected by one virus has been reported by others.[15,18-21] This observation is in line with studies documenting that co-infections are not often observed in mechanically ventilated children.[17, 22]

Disease severity varied with the type of viruses and specific virus pairs involved in co-infection. For example, in studies where RSV/Bocavirus and Influenza A/Coronavirus co-infections were frequently observed, a significantly increased risk for hospitalization was reported[23, 24], whereas in studies where RSV/Adenovirus and RSV/Rhinovirus co-infections were seen, a reduced risk of hospitalization was reported.[20, 25] Future studies should employ stratified analyses on the effect of co-infections on disease outcome to elucidate which combination of viruses increase or decrease disease severity.

The detection of viruses by RT-PCR does not prove per se a causal relationship. The development of sensitive molecular assays has increased the number of viruses detected in respiratory samples compared with conventional methods. The detection of viral nucleic acids by RT-PCR can reflect either past infection, an asymptomatic or asymptomatic infection, or incipient infection in which symptoms have not yet developed. The high viral detection rates in asymptomatic children indicate that the presence of viral or bacterial genomes is often not associated with disease.[26,27] The distinction between co-infection and co-detection has been ignored in most studies and may be particularly relevant in cases with multiple viral infections in which one has to distinguish which virus is the cause of disease symptoms. Epidemiologic studies with both quantitative PCR and serologic studies may be of additional value to demonstrate causality between viral detection and clinical symptoms for some viruses, especially when low copy numbers and multiple viruses are detected.

Quantitative PCR techniques can, in theory, help identify the agent responsible for symptoms based on high acute-phase viral replication. qPCR derived Ct values are an indirect measure of viral load, representing the number of PCR cycles necessary to pass the detection treshold. Although lower Ct values are more likely to suggest a virus as a true cause of acute lower respiratory tract infection, Ct values of different viruses are difficult to compare.[28, 29]

Virus-virus interactions may influence the host immune response resulting in reduced virulence of other respiratory viruses. Greer et al. described a potential protective effect of the presence of RV in the nasopharynx on new viral infections by the induction of interferon stimulated genes resulting in less severe disease or less co-infections.[30] Chorazy et al. reported that children with viral co-infections were less likely to be admitted to the ICU than children with single virus infections.[21] As this seemingly protective effect of multiple infections was

unexpected, they performed secondary analyses to virus-positive, hospitalized children with confirmed or suspected acute respiratory infections (ARIs) for whom a bacterial test had been ordered during the same hospitalization. Children with virus–bacterial co-infection, as compared with children with single virus infection, were more likely to be admitted to an ICU.[21] This may indicate that undetected bacterial co-infections play a role in severe infections caused by a single virus.
In conclusion, the precise mechanisms of a potentially protective effect of multiple viral infections are not yet well understood and more research is needed to understand the processes involved in respiratory viral co-infections.

Viral Load

The use of qRT-PCR derived Ct values have allowed an accurate estimation of the viral load in clinical samples. Some studies have associated viral load with disease severity and failed to establish a causal relationship.[31, 32] Other studies, however, revealed a significant association between disease severity and viral load.[18, 33-36]
One of the confounding factors on the association between viral load and disease severity is that most studies measure Ct values at one time point. Viral load is a dynamic process requiring longitudinal measurements to obtain optimal interpretation. Studies that measured viral load longitudinally and adjusted for disease duration demonstrate that increased viral load and delayed viral clearance is associated with increased markers of disease severity in young RSV infected children.[33, 34, 37, 38] An association between temporal dynamics of viral load and clinical symptoms may have important therapeutic implications, supporting a potential clinical benefit of RSV antiviral drugs.

II HOST IMMUNE RESPONSE

An improved understanding of the interactions between virus and host immune response is important to better understand factors and mechanisms determining disease severity. This may also facilitate the identification of biomarkers that can predict clinical outcome.
Virus specific structural components are recognized by pattern recognition receptors of the host upon RSV infection of airway epithelial cells. Activation of many of these receptors causes up-regulation and expression of several pro-inflammatory genes in the lung. This leads to recruitment and activation of

different leukocytes subsets (monocytes, macrophages, neutrophils, T-cells, NK-cells), and contributes to the production of inflammatory factors (chemokines and cytokines). These in turn suppress viral replication and eliminate the virus but simultaneously contribute to tissue damage.

Systemic Immune Response upon RSV Infection

A number of clinical studies have, in line with our results, documented an association between RSV disease severity and changes in concentrations of inflammatory mediators in blood including interleukin (IL)-6, IL-8, Granulocyte-colony stimulating factor (G-CSF) and Chemokine (C-C motif) ligand 5 (CCL-5). Several mechanisms may be involved in the systemic inflammatory response following local airway inflammation. Inflammatory mediators generated in the airways translocate into the circulation, following a natural gradient (due to large amounts of inflammatory molecules in the lung) or as a result of increased permeability of the capillary bed vascular system which often accompanies the lung inflammatory process. In addition, triggers of lung inflammation, such as ultrafine particulate matter, LPS and other toxins, translocate from the airspaces to the bloodstream, either directly contributing to the systemic response or stimulating circulating immune cells such as monocytes to produce pro-inflammatory mediators that contribute to the systemic response. Another mechanism is direct infection of circulating cells followed by a systemic inflammatory response. Although RSV is assumed to primarily infect epithelial cells, RSV RNA has been demonstrated in whole blood, and RSV antigens and RNA have been found in circulating blood mononuclear leukocytes blood and lung neutrophils.[39-43] In addition, in a murine model an association between the time course of RSV RNA detected in blood and severity of symptoms has been demonstrated.[44] Viral particles may be released from infected airway epithelial cells and then enter the circulation or circulating cells may take up the virus in the airway lumen and then recirculate back into the bloodstream.

Lymphocytopenia in Severe RSV Infection

Severe RSV infections in young children are associated with lower peripheral T-cell and NK-cell counts.[45-48] The role of lymphocytopenia in disease severity is further supported by observations in an experimentally human challenge model for RSV infection. Marked differences in subpopulations of white blood cells between symptomatic and asymptomatic individuals over time were observed.[49]

RSV-infected subjects developed a relative lymphocytopenia, monocytosis and neutrophilia, which closely corresponds with the increase and decrease of clinical symptoms.[54]

The observed lymphocytopenia may be explained in several ways. Lymphocytopenia can be the result of accumulation of inflammatory cells, such as lymphocytes, at the site of infection. However, although primary RSV infection in mice produces a strong lymphocyte infiltration into the lungs, Welliver et al. showed a lack of lymphocytes in the lungs of children who died from severe RSV bronchiolitis.[49]

Lymphocyte apoptosis may be another mechanism for RSV-induced lymphocytopenia. Roe et al. demonstrated up-regulated expression of cell surface receptors involved in apoptosis (Fas and TRAIL receptor) on CD4+ and CD8+ lymphocytes and increased plasma levels of soluble Fas ligand during acute illness upon RSV bronchiolitis.[46]

A third explanation for lymphocytopenia is a decreased capacity to induce T-cell proliferation upon RSV infection. Direct interaction between the virus and T-cells, inhibition of T cells by infected antigen presenting cells or a decrease in expression of HLA-DR can result in the inhibition of T-cell proliferation. Low T-cell proliferative responses and interferon (IFN)-γ production upon RSV infection have been described and suggest a role of lymphocyte suppression in the pathogenesis of severe RSV infection.[50-52] Mejias et al. found suppression of T and B-cell related genes in children with RSV bronchiolitis using gene expression profiles of whole blood samples.[53]

Our microarray analyses showed an upregulation of activation markers on PBMCs in the severe group and lack of apoptosis. This may suggest that the lymphopenia results from recruitment of lymphocytes to the site of infection. More accurate measurements at the site of infection are needed to elucidate this.

Implications for the Future

Mechanisms which contribute to RSV pathogenesis include direct viral cytopathology, exaggerated cytotoxic lymhocytes responses, imbalanced CD4+ T cell responses, excessive inflammatory responses, and altered immune responses due to young age.[55, 56] In most cases, however, there is not a single causative factor. This may have important consequences for therapy. For example, antiviral therapy will not be effective in individuals with an excessive immune response and anti-inflammatory therapy may result in further clinical deterioration in the individual in which viral induced damage dominates.

A comprehensive approach is required to understand the complex biological interactions between virus and host in the clinical context, and how this contributes to disease severity. The research approach should include multifaceted assays that simultaneously (1) examine the virus dynamics, (2) assess innate and adaptive immune responses, and (3) interpret the obtained data for integrated bioinformatic analyses.
Oshansky et al. provide a model example how to approach such an investigation in children with influenza infection.[57] They obtained sequential samples in two different compartments (blood and nasal lavages) in a cohort of patients of different ages and severity. To identify markers of disease severity, they measured a number of virology and immune markers using multifaceted molecular and cellular assays. Data were analyzed using state of the art bioinformatic tools. This comprehensive strategy revealed an innate profile that correlated with disease progression independent of viral dynamics and age.
Using such a "system analysis" approach will capture the complexity of the host–virus interaction and accelerate the identification of biomarkers that can objectively predict clinical outcome.

III TRANSCRIPTOMICS AND PROTEOMICS

Transcriptomics

With the use of molecular platforms, such as micro-array, it has become possible to measure gene-expression profiles of the genome. This enables to link genes or clusters of genes (pathways or modules) to disease. Data on blood gene expression profiling in children with RSV bronchiolitis are still limited. The studies that have been published on this subject indicate that gene expression profiles from children with acute RSV bronchiolitis can be discriminated from healthy controls and allow to discriminate between viral and bacterial infections.[58-60]
Mejias et al. were the first to publish a comprehensive analysis of whole blood gene expression profiles in infants with RSV LRTI. They described a significant association between gene expression profiles and clinical disease severity of acute viral LRTIs.[53] In addition a genomic score was developed based on 1,536 significantly differentially expressed transcripts that correlated with disease severity. We used another approach and performed a Prediction Analysis of Microarrays (PAM), based on bayesian nonparametric modeling, to identify the minimum number of genes that are required to distinguish mild from severe

RSV infection. By doing so, we were able to identify olfactomedin-4 (OLFM4) as a potential biomarker for disease severity.

To date, most micro-array studies on viral respiratory tract infections are performed on whole blood samples. One of the disadvantages of using these samples is the uncertainty whether the observed differences in gene expression reflect changes in transcriptional activity or an altered cellular composition of blood samples.

The strength of using isolated cell populations for gene-expression studies is that it provides a cell-specific RNA source thereby minimizing confounding data due to heterogeneous cell populations. Earlier micro-array studies, including ours, reveal that blood RNA profiling is certainly more than just an expensive blood count: results depend not only on the number and type of cells in the blood sample, but also on the activation state and the exposure of the cells to stimulatory or inhibitory factors in the blood or in the tissues through which the cells have passed.[53]

These micro-array techniques can also be used to obtain more detailed information on the mechanism of disease severity. We performed microarray analyses on different PBMC subpopulations (T-cells, B-cells, monocytes and NK-cells) providing cell-specific information on gene expression. These data are currently functionally analysed more in depth. This analysis seeks to compare gene expression data from PBMCs, neutrophils and PBMC subpopulations with existing gene expression data in order to obtain more insight in pathogenesis of viral respiratory tract infections.

Proteomics

Proteomic technologies are a tool to study the pathogenesis of infectious diseases in both experimental and clinical models. The advantage of this approach is that it enables the identification of novel key proteins involved in the pathogenesis of severe RSV infection.

Transcriptome studies have improved our understanding of host cell responses to RSV.[61-65] However, mRNA expression levels cannot be extrapolated to structure and function of proteins. Translational and post-translational modifications affect not only the level of a mature protein but also its function. The effects on protein structure and biological function of alternative splicing, post-translational modifications and virus–host interactions cannot be easily predicted by transcriptomics. Another advantage of application of proteome analyses is that proteins can be measured in many body fluids while gene expression profiling is

dependent on the presence of nucleated cells.
Viral replication and propagation, but also the host immune response cause host cellular proteome variations. One of the most promising applications of proteomics is the identification of protein targets for biomarkers, vaccination or new treatment strategies.
In addition, we have shown in chapter 8 of this thesis that proteomic technologies can be used to elucidate the molecular mechanism underlying vaccine induced RSV specific enhanced disease providing an unbiased overview of these effects and to identify protein key players/ biomarkes of enhanced disease. The identified protein biomarkers can be used to support the development as well as the detailed evaluation of next generation RSV treatment strategies and vaccines.
Most proteome studies investigating RSV infection have been carried out in cell lines.[66-68] Thus far, the number of clinical studies using proteome analyses in children with viral lower respiratory tract infections is limited. Pilot experiments show that the protein composition in nasal secretions from children with viral LRTIs is different between healthy children and children with viral infections.[69, 70] These findings illustrate that proteomics can be used to identify new markers for viral respiratory infection. The identification of a specific proteomic fingerprint in adult SARS patients forms an interesting example for the diagnostic and prognostic use of proteome analyses.[71-73]
Integration of multiple –omics techniques may provide further understanding of mechanisms of infection. In our studies, we did not correlate proteomics findings with gene expression profiles. Cellular concentrations of protein do not strongly correlate with the abundance of their corresponding mRNAs. It has been estimated that about 40% of the variation in protein concentration can be explained by knowing mRNA abundance.[74, 75] Biological explanations such as regulatory mechanisms at the levels of transcription, post-transcriptional and post-translational modifications and technical issues contribute to the poor correlation between transcriptomic and proteomic expression data.

Implications for the Future

Linking gene expression, protein expression and other –omics technologies such as metabolomics are important steps to obtain a better understanding of the pathogenesis of infectious disease and provide new targets for biomarker development, treatment strategies and vaccine development. It is important to integrate mRNA and protein expression data into a common framework, to scale and merge the data together, and to analyze changes in categories such as

functional protein class, subcellular localization, and secondary structure. This multisystem approach is depending on the development of new bio-informatics tools and close collaboration of clinicians, biologists and bioinformaticians.
The next challenge is to bridge the gap between identification of genes and proteins that may serve as biomarker or targets for vaccine development and treatment strategies and clinical validation. This is a complicated process requiring integration of research findings within worldwide databases. In addition, for actual bed-side translation of findings obtained from proteomic studies, large clinical multicenter studies for validation have to be performed.[76]

IV BIOMARKERS

In chapter 1 we reviewed the state of art of biomarkers in pediatric infectious diseases. We concluded that gene expression profiles can be used, either directly or indirectly, to identify causative pathogens and discriminate between bacterial infections, infections caused by other pathogens, such as viruses, and non-infectious causes of fever.[77] However, this review was published in 2006 and since that time much progress has been made. In the following section we provide a short update on this topic.

Transcriptomics

Zaas et al. provided a novel approach for diagnosis of acute viral respiratory infections based on blood transcriptional profiles from experimentally infected and uninfected subjects. They identified an ''acute viral respiratory signature'' of 30 genes, that clearly distinguished symptomatic infected individuals (with influenza A, rhinovirus, and RSV) from asymptomatic uninfected individuals.[60] This signature contained genes that were shared by all three viral infections. They also identified genes that were more specific for each viral infection providing further evidence of the potential value of this approach to define diagnostic signatures that are common for a group of pathogens and those that are pathogen-specific.
To simplify the complex micro array analyses, Chaussabel et al. developed a novel data mining algorithm of blood transcriptional modules based on sets of genes that follow similar expression patterns in large numbers of samples. They showed that different infectious diseases are characterized by a unique modular combination generating a characteristic disease fingerprint.[78] Subsequently,

clinical markers of disease severity were correlated with changes in host gene expression. Transcriptional profiling provides a comprehensive assessment of the host response. This allows correlating clinical markers of disease severity with changes in host gene expression. To this purpose these investigators developed genomic severity score that can help classifying patients according to clinical characteristics.[78]
In a recent study a molecular distance to health (MDTH) score was used in a cohort of adult patients with tuberculosis. The MDTH score represents the 'molecular distance' of a given sample relative to a baseline (for example, healthy controls). The number of standard deviations for all qualifying genes is added to yield a single value, the global distance of the sample from the baseline.. This score allowed to classify patients according to clinical characteristics and to estimate disease severity.[79, 80] In addition, these scores can be used to monitor treatment, since MDTH scores progressively decreased and normalized during treatment.
Another application of genome wide techniques is to identify the disease causing micro-organism in clinical samples with multiple micro-organisms. Based on host immune responses these techniques can determine whether the microbe is causing the disease or if it reflects colonization and/or asymptomatic shedding. These assays will be extremely valuable to study dual or triple viral co-infections as well as viral-bacterial co-infections. At this moment a golden standard to discriminate viral from bacterial respiratory tract infections is lacking, resulting in inappropriate use of antibiotics.

Proteomics

While important steps have been taken in the exploration of clinical applications of gene expression profiles, actual bed-side translation of the findings obtained from proteomic research has thus far been limited. This may be explained by the fact that most proteome techniques are relatively low throughput and too time consuming to implement in the clinic. Therefore most proteome studies include small numbers of patients and do not have enough statistical power. In addition, validated methods to reduce the large numbers of differentially expressed proteins to a small number of clinical relevant proteins for further validation are not yet available.
In the future, a systems biology approach to viral LRTIs, which combines different "omics" tools such as genomics, transcriptomics, proteomics, metabolomics may facilitate further understanding of the mechanisms of infection and contribute to find targets for prognostic biomarkers and new treatment strategies. However,

better informatics methods are needed to bridge the gap between biomarker discovery and validation.[76]

Implications for the Future

Despite the global health impact of viral respiratory infections, and RSV specifically, no accurate virology or immune markers to predict clinical outcome are available. Combining several biomarkers into a single classification rule may help to improve accuracy and, therefore, clinical usefulness. After validation of the potential markers in large clinical sample sets, multiple biomarkers have to be tested simultaneously to assess their effectiveness in various combinations and improve both sensitivity and specificity. In this thesis we show that a combination of heterogeneous markers can distinguish disease severity in young RSV-infected children with a higher sensitivity and specificity compared with individual markers. Such combinations of multiple biomarkers should be utilized in the development of multimarker panels. Markers from different sources can be combined and should include clinical parameters, molecular biomarkers and biochemical biomarkers.

At this moment, transcription biomarkers are not available for clinical use since processing is too time consuming, expensive and not standardized compared. However, novel and affordable PCR-based tools with faster turnaround time are currently under development. This will facilitate the application of gene expression profiles 'bed side' in the future.[81]

Blood transcriptome analysis has been shown to be useful for the detection of new biomarkers for LRTIs. In addition to the blood profiling approach, improved genomic approaches that use high-throughput molecular and cellular profiling of cells in different clinical subsets will further contribute to biomarker discovery.

Current efforts to discover and develop severity-related biomarkers may contribute to a more optimal clinical decision-making. Once new treatment strategies will become available, translation of biomarkers into the clinic will lead to implementation of personalized therapies that may benefit patients with LRTIs. In order to achieve this goal, well-designed large-scale multi-center profiling studies should be designed to discover new biomarkers. Extensive cellular and molecular profiling of human subjects will generate enormous amounts of data. Therefore, efficient data management, advanced bioinformatic tools and statistical methods for integrated analyses are essential to provide the full potential of high throughput profiling approaches in patients with LRTIs.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis we identified a number of inflammatory markers to discriminate between mild and severe manifestations of viral bronchiolitis in young children. Objective assessment of clinical severity of RSV infection in young children may be improved by including combinations of immunological parameters. Most of the results of the studies in this thesis need to be validated in independent cohorts to establish their clinical value. It is tempting to speculate that inflammatory markers may precede clinical symptoms and can be used early in the course of disease to predict severe outcome. However, the potentially predictive value of these markers needs to be further investigated in the future.

We propose that a combination of inflammatory markers in clinical prediction tools can improve patient care and reduce hospitalization rates. We have already shown that using linear regression tools new markers can be added to optimize prediction rules (Ahout et al. submitted). It would be of great clinical importance to select those patients that will not develop a severe course of disease and won't require any supportive interventions. This may reduce unnecessary hospitalizations and result in less healthcare costs. For this purpose, prognostic biomarkers may have an important clinical value in addition to clinical parameters. This is especially relevant in infants below the age of 3 months.

Early identification of patients that will develop a severe course of disease may help general practitioners and pediatricians to target individualized monitor or treatment strategies. Biomarkers may become a valuable clinical tool once newly developed treatment strategies for viral bronchiolitis become available in the future. Interestingly, a recent paper reported preliminary data on the antiviral activity of a fusion inhibitor, GS-5806, in modulating experimental RSV infection in adults.[82] Treatment with this fusion inhibitor resulted in reduction of viral load and symptom scores. The clinical efficacy of such small molecule antiviral agents needs to be assessed in children with natural infections. Until that time, biomarkers can also be used for research purposes to stratify patients in clinical studies, to study effects of interventions, such as treatment, and as secondary end-points in intervention studies.

Further larger scale studies are required to evaluate the predictive value of co-infections and viral load dynamics on the clinical course of bronchiolitis. These findings are important because they debate the additional value of viral diagnostics in clinical prediction models for viral bronchiolitis. At this moment, the clinical utility of viral testing in cases of bronchiolitis concerning management decisions or clinical outcomes is limited and in the available guidelines viral testing is not

recommended for bronchiolitis from a cost-effectiveness perspective.[83]
Understanding the complex interactions between the virus and the host, and how it defines disease severity, requires a system analysis strategy. This strategy should include assays that simultaneously examine viral dynamics and innate and adaptive immune responses by employing different 'omics' tools such as genomics, transcriptomics, proteomics, and metabolomics. This system biology approach has to be combined with sophisticated tools for large data analyses. Such an approach will facilitate further understanding of mechanisms of infection but will also elucidate new targets for prognostic biomarkers and new treatment strategies resulting in better patient treatment. Finally, we expect that ultimately an integrative personal omics profile (iPOP) will contribute to personalized medicine in both health and disease.[84]

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Chapter 10

Summary

SUMMARY

In this thesis we 1) evaluated the viral-host response in young children with viral lower respiratory tract infections (LRTIs), with a focus on respiratory syncytial virus (RSV) infections; and 2) identified potential biomarkers to assess disease severity in young children with viral LRTIs.

In **chapter 2** the use of biomarkers in the field of pediatric infectious diseases was reviewed. The development of diagnostic biomarkers may lead a to more rapid diagnosis, more reliable discrimination between infectious and non-infectious diseases, improved management, better outcomes, and a decrease in inappropriate use of antibiotics. With the development of transcriptomic and proteomic technologies, host immune responses can be unraveled thus contributing to better diagnostics and disease monitoring as well as predicting the individual's susceptibility to disease and response to medical therapy. Although promising, the clinical application of these technologies is still limited at present.

Children with bronchiolitis show a huge variability in disease severity. Disease severity is the result of a dynamic interplay between both host and viral factors. In **chapter 3** we evaluated the association between the detection of multiple viruses by RT-PCR and disease severity in young children with bronchiolitis. We showed that the detection of more than one virus is not associated with increased disease severity in children with bronchiolitis. Remarkably, in children older than 3 months we found an association between more severe disease and RSV mono-infections. In addition, we found that viral load in children with single RSV infections was not associated with disease severity. We concluded that disease severity in children with bronchiolitis is not associated with infection by multiple viruses and that other factors, such as age or immune response are more important contributors to disease severity.

Subsequently, searching for biomarkers for disease severity, we evaluated several inflammatory parameters involved in the host immune response. In **chapter 4**, we investigated the role of MMP-8 and MMP-9 as markers for disease severity in children with viral LRTIs. Disease severity of viral LRTIs in children was associated with increased gene expression levels, but not with plasma (protein) concentrations, of MMP-8 and MMP-9 in both PBMCs and granulocytes.

In **chapter 5**, we showed that severe RSV infection was characterized by young age, lymphocytopenia, increased IL-8, granulocyte colony-stimulating factor (G-CSF) and IL-6, and decreased chemokine (C-C motif) ligand (CCL-5) concentrations in plasma. The combination of IL-8 and CCL-5 plasma levels and CD4+ T-cell counts discriminated children with severe from those with mild RSV infection with 82% sensitivity and 96% specificity. From these results it can be concluded that the use of combinations of inflammatory markers has additional value in assessing severity of RSV infection and may guide clinical management in the future.

In **chapter 6** we showed that combining genome-wide expression profiling of blood cell subsets with clinically well-annotated samples can lead to identification of new biomarkers for disease severity of viral RTI. We identified a single gene, Olfactomedin-4 (OFLM4) that allowed us to distinguish patients with severe disease from those with mild disease. Patients with a high OLFM4 gene expression level were more likely to develop severe disease, after correction for age at hospitalization and gestational age. These results were validated in a new patient cohort and this confirmed OLFM4 in PBMC as a marker for disease severity in children with viral lower RTIs.
The results of this study and the ones presented in chapter 4 and 5 support the hypothesis that differential expression of genes involved in the immune response underlies the severity of RSV infections and that measurement of these parameters may improve current tools to predict severity of RSV infection.

In **chapter 7** we used an unbiased approach with proteomic technologies to identify targets for the development of biomarkers, future therapeutics or prevention strategies against severe RSV infection. We used human airway epithelial cells (A549) infected with RSV, human metapneumovirus (hMPV), parainfluenzavirus (PIV) type 3 and measles virus (MV) to identify specific and common host response markers by 2-D DIGE analyses. Cells infected with RSV or hMPV showed more changes in protein expression in comparison with PIV and MV-infected cells. The expression of proteins involved in the induction of apoptosis upon infection was differentially altered by the four viruses. Some of the identified proteins have previously been described for viral induced apoptosis by non-respiratory viruses. In addition, we were also able to identify novel proteins altered by respiratory viral infection. In conclusion, this study shows that proteomic techniques, like the 2-D DIGE analysis, can be used to identify proteins that are altered by respiratory viral infections which may serve as new targets for future therapeutic modalities.

In **chapter 8** we present the results of a study based on the important observation from trials with a formalin inactivated experimental vaccine in the 1960s indicating that severe disease manifestations during RSV infection would, at least partially, be caused by a vigorous immune response. The vaccination resulted in augmented disease upon subsequent natural RSV infection in vaccinated infants as compared to controls. To identify protein markers and regulatory mechanisms that are associated with or are underlying these vaccine induced aberrant host responses, we used an in vivo mouse model of vaccine enhanced disease and applied mass spectrometry assisted protein profiling. Using a comparative proteomics approach seven proteins were identified as biomarkers of enhanced disease (Epx, Chil3, Itgam, Arg1, Clca3, PurB and Hk3). These proteins included proteins specific for the infiltrating eosinophils but also proteins that are postulated to play a role in the preceding stages of induction and establishment of skewed vaccine induced hypersensitivity responses.

In **chapter 9** we reflect on the results of our studies and clinical implications and recommendations for future research are formulated. In the future, a system analysis approach using different -omics technologies combined with sophisticated tools for large data analyses will facilitate further understanding of mechanisms of infection and elucidate new targets for prognostic biomarkers and new treatment strategies resulting in better patient treatment.

Addendum

NEDERLANDSE SAMENVATTING

Inleiding

De onderzoeken in dit proefschrift richten zich op ernst van ziekte bij kinderen met virale onderste luchtweginfecties (OLWI), met een focus op luchtweginfecties veroorzaakt door respiratoir syncytieel virus (RSV).
RSV is een veel voorkomende oorzaak van luchtweginfecties en een belangrijke reden voor ziekenhuisopnames bij jonge kinderen. Klachten van een luchtweginfectie veroorzaakt door RSV variëren van milde bovenste luchtwegklachten tot ernstige onderste luchtweginfecties die in sommige gevallen leiden tot respiratoir falen waarvoor beademing op een intensive care nodig is. De meeste kinderen hebben tenminste één RSV infectie doorgemaakt voor de leeftijd van 2 jaar. In Nederland worden jaarlijks ongeveer 1500-2000 kinderen onder de leeftijd van 1 jaar opgenomen en hiervan worden 100-150 kinderen beademd op een intensive care afdeling.
Er zijn verschillende risicofactoren (zoals prematuriteit, aangeboren hartafwijkingen en chronische longziekten) die kunnen leiden tot een ernstiger beloop van RSV infecties. Echter, meer dan de helft van de kinderen die opgenomen wordt op een intensive care afdeling was van tevoren goed gezond. Het is niet duidelijk waarom juist deze kinderen een ernstiger beloop ontwikkelen. Momenteel is het voor artsen moeilijk in te schatten welke kinderen ernstiger ziek zullen worden. Bij ongeveer 35% van de kinderen die opgenomen wordt vinden geen ondersteunende interventies plaats gedurende de opname. Daarnaast wordt 5-7% van de kinderen die beoordeeld worden in het ziekenhuis later in het ziektebeloop alsnog opgenomen. Het ontwikkelen van markers in bloed of luchtwegmaterialen die ernst van ziekte kunnen voorspellen zouden in belangrijke mate artsen kunnen helpen in de beslissing kinderen op te nemen danwel naar huis te ontslaan. Hiermee kunnen enerzijds kinderen geselecteerd worden die nauw geobserveerd moeten worden gedurende het ziektebeloop en anderzijds onnodige opnames voorkomen worden.

De onderzoeken die beschreven zijn in dit proefschrift dienen 2 hoofddoelen:

1. Evalueren van de interacties tussen gastheer en virus in jonge kinderen met virale OLWI's, met een focus op RSV infecties
2. Identificeren van potentiële biomarkers die ernst van ziekte kunnen vaststellen in jonge kinderen met virale OLWI's

In de afzonderlijke hoofdstukken worden de verschillende onderzoeken beschreven.

Hoofdstuk 2 geeft een overzicht van het gebruik van biomarkers in de pediatrische infectieziekten. Diagnostische biomarkers kunnen bijdragen aan een snellere diagnose, beter onderscheid tussen infectieuze en niet-infectieuze oorzaken, betere behandeling en uitkomsten van ziekte, en een afname in het onnodig gebruik van antibiotica. De ontwikkeling van transcriptomics en proteomics technieken, waarmee de afweerreactie van de gastheer op een microorganisme op respectievelijk genexpressie- en eiwitniveau onderzocht kan worden, draagt bij aan betere diagnostische mogelijkheden, monitoring, als ook het voorspellen van de vatbaarheid van een individu om een bepaalde ziekte te krijgen en het voorspellen van de reactie van een individu op een bepaalde behandeling. Ook al klinkt dit veelbelovend, de klinische toepassing van dergelijke technieken is momenteel nog zeer beperkt.

Kinderen met RSV infecties laten veel variatie zien in ernst van ziekte. Ernst van ziekte is het gevolg van de interactie tussen virale factoren en gastheerfactoren. **Hoofdstuk 3** beschrijft de relatie tussen de detectie van meerdere virale pathogenen in neusspoelsel en ernst van ziekte in jonge kinderen met bronchiolitis. De detectie van meer dan 1 virus in het neusspoelsel van kinderen was niet geassocieerd met toegenomen ernst van ziekte. Kinderen ouder dan 3 maanden met RSV mono-infecties hadden zelfs een ernstiger ziektebeloop dan kinderen bij wie meerdere virussen tegelijkertijd aangetoond waren. Deze resultaten suggereren dat ernst van ziekte in kinderen met bronchiolitis niet geassocieerd is met de detectie van meerdere virale pathogenen, maar dat andere factoren zoals leeftijd of afweerreactie een belangrijke rol spelen.

In de zoektocht naar biomarkers voor ernst van ziekte werden vervolgens verschillende inflammatoire markers, betrokken in de afweerreactie op virussen, geëvalueerd. In **hoofdstuk 4** onderzochten we de rol van matrix metalloproteinases (MMP)-8 en MMP-9 als markers voor ernst van ziekte in kinderen met virale OLWI's. Kinderen met een ernstig beloop van een virale OLWI hadden een hogere genexpressie van MMP-8 en MMP-9 in perifere mononucleaire bloedcellen (PBMCs) en granulocyten dan kinderen met een milder beloop. Plasmaconcentraties van MMP-8 en MMP-9 lieten deze associatie niet zien.

In **hoofdstuk 5** worden verschillende klinische en inflammatoire parameters vergeleken in kinderen met een mild, matig of ernstig ziektebeloop. De combinatie van IL-8 en CCL-5 plasma concentraties en CD4+ T-celaantallen kon kinderen met ernstige ziektesymptomen onderscheiden van die met milde symptomen met een sensitiviteit van 82% en een specificiteit van 96%. Op basis van deze bevindingen concluderen wij dat het gebruik van combinaties van inflammatoire markers van toegevoegde waarde kunnen zijn in het vaststellen van ernst van RSV infectie. Dit zou in de toekomst kunnen bijdragen aan klinische beslissingen.

Hoofdstuk 6 laat zien dat het vergelijken van genexpressieprofielen van verschillende bloedcelpopulaties van kinderen met verschillende mate van ernst van ziekte kan leiden tot de identificatie van nieuwe biomarkers voor ernst van ziekte. Eén enkel gen, Olfactomedine-4 (OLFM4), kon het onderscheid maken tussen kinderen met ernstige ziekte en die met een milder beloop. Ook na correctie voor leeftijd en zwangerschapsduur hadden kinderen met een hoog OLFM4 genexpressie niveau vaker een ernstiger ziekte beloop dan kinderen met een laag OLFM4. Validatie in een nieuw patiëntencohort heeft bevestigd dat OLFM4 een mogelijke marker is voor ernst van ziekte in kinderen met virale OLWI's.
De resultaten van deze laatste studie en die van de studies beschreven in hoofdstuk 4 en 5 ondersteunen de hypothese dat verschillen in genexpressie en plasmaconcentraties van inflammatoire markers ernst van ziekte kunnen reflecteren. Het meten van dergelijke markers kan de huidige beschikbare methodes voor het inschatten en eventueel voorspellen van ernst van RSV infecties verbeteren.

Hoofdstuk 7 beschrijft een studie waarin proteomics technieken gebruikt worden om eiwitten te identificeren die kunnen dienen als biomarkers, of als kandidaat voor toekomstige behandelingsmogelijkheden en preventieve strategieën van ernstige RSV infecties. Hiervoor werden humane luchtwegepitheelcellen geïnfecteerd met verschillende virussen waaronder RSV met als doel algemene en specifieke gastheer respons markers te identificeren met de 2-D DIGE methode. Dit is een vorm van gel elektroforese waarbij uit verschillende eiwitsamples eiwitten worden gescheiden op basis van grootte en isoelektrisch punt. Omdat verschillende samples gelabeld zijn met een verschillende fluorescerende kleur kunnen verschillen in aanwezigheid en hoeveelheid van bepaalde eiwitten in verschillende samples onderscheiden worden.
In deze experimenten toonden cellen geïnfecteerd met RSV meer veranderingen

in eiwitexpressie vergeleken met cellen geïnfecteerd met een aantal andere virussen. De expressie van zowel bekende als nog onbekende eiwitten betrokken bij de inductie van geprogrammeerde celdood bleek significant verschillend ten opzichte van controles. Dergelijke eiwitten kunnen een mogelijk nieuw target zijn voor toekomstige therapeutische of preventieve strategieën.

Eerdere pogingen om een vaccin tegen RSV te ontwikkelen mislukten. In een vaccinatie experiment in de 1960s resulteerde vaccinatie bij kinderen in een ernstiger beloop van een daaropvolgende natuurlijke RSV infectie vergeleken met niet-gevaccineerde kinderen. Meerdere onderzoeken hebben aangetoond dat dit ging om een excessieve immuunrespons, echter de precieze aard van deze respons is vooralsnog onduidelijk. In **hoofdstuk 8** onderzochten wij deze door vaccinatie geïnduceerde immuunrespons in een in vivo muizen vaccinatiemodel met behulp van op massa spectrometrie gebaseerde proteomics technieken. We identificeerden 7 eiwitten die kunnen dienen als marker van de door vaccinatie geinduceerde excessieve immuunrespons.

In **hoofdstuk 9** reflecteren we op de resultaten van de afzonderlijke studies. Onze belangrijkste bevindingen zijn:

1. Ernst van ziekte in jonge kinderen met een virale OLWI is niet geassocieerd met de detectie van meerdere virussen tegelijkertijd of met de virale load
2. Verschillende inflammatoire markers gemeten op genexpressie niveau (MMP-8 en MMP-9) of in plasma (combinatie van IL-8 en CCL-5 plasmaconcentraties en CD4+ T-cel aantallen) kunnen ernstige van milde RSV infecties onderscheiden
3. Transcriptoom analyses van patiëntmateriaal heeft geleid tot de identificatie van nieuwe biomarkers voor ernst van ziekte (OLFM4)
4. Proteoom analyses van in vitro en in vivo (muizen) modellen hebben meerdere gastheer eiwitten aangetoond die betrokken zijn bij de immuunrespons

De geïdentificeerde markers dienen gevalideerd te worden in onafhankelijke patiëntcohorten om de klinische waarde te bepalen. Daarnaast zal de voorspellende waarde van deze markers onderzocht moeten worden. Toekomstig onderzoek moet zich richten op geïntegreerde analyses van virale dynamiek en gastheer immuunrespons door middel van het combineren van verschillende –omics technieken met geavanceerde data analyses. Deze benadering middels multisysteem analyse zal de identificatie van nieuwe prognostische biomarkers

en aanknopingspunten voor behandelingsmogelijkheden en vaccinatie mogelijk maken.
Indien in de toekomst nieuwe behandelingsstrategieën voor RSV infectie beschikbaar komen, worden dergelijke biomarkers een waardevol klinisch instrument. Toepassing van dergelijke markers in klinische predictiemodellen kan bijdragen aan een verbetering van patiëntenzorg en reductie van gezondheidszorgkosten.

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CURRICULUM VITAE

Kim Brand werd op 1 juli 1980 geboren in Schiedam. Zij behaalde in 1998 haar VWO diploma, waarna ze geneeskunde ging studeren aan de Erasmus Universiteit te Rotterdam. In 2004 legde zij cum laude haar artsexamen af. Daarna heeft zij 1 jaar als arts-assistent niet in opleiding op de afdeling kindergeneeskunde gewerkt in het voormalig Medisch Centrum Rijnmond-Zuid locatie Zuider (opleider prof. dr. Oudesluys-Murphy) te Rotterdam, tegenwoordig het Maasstadziekenhuis. In augustus 2005 begon zij aan het promotietraject van dit proefschrift en de opleiding tot kinderarts in het Radboud Universitair Medisch Centrum (opleider prof. dr. L. Kollee, vanaf 2007 dr. J.M.T. Draaisma) te Nijmegen. De perifere stage Kindergeneeskunde volgde zij in het Canisius Wilhelmina Ziekenhuis te Nijmegen (opleider dr. B. Semmekrot). Onder leiding van prof. dr. R. de Groot, hoofd van de afdeling Kindergeneeskunde, en prof. dr. P.W.M. Hermans heeft zij op het Laboratorium Kindergeneeskunde Infectieziekten gewerkt aan de voltooiing van dit proefschrift.
Kim Brand woont samen met Maarten Truijers. Zij hebben samen twee zoons, Hugo (2011) en Jidde (2013). In mei 2015 zullen zij naar Rotterdam verhuizen waar Kim de opleiding tot Kinderallergoloog zal volgen in het Erasmus MC-Sophia Kinderziekenhuis.

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LIST OF ABBREVIATIONS

AdV	adenovirus
APC	allophycocyanin
β-ME	β-mercapto-ethanol
BSA	bovine serum albumin
CCL	chemokine (C-C motif) ligand
CoV	coronavirus
Ct	cycle treshold
CXCL	chemokine CXC ligand
(2)D DIGE	2 dimensional gel electrophoresis
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
EV	enterovirus
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
hBoV	human bocavirus
hMPV	human metapneumovirus
IFN	interferon
IL	interleukin
IP	inducible protein
IQR	interquartile range
IV	influenza virus
LRTI	lower respiratory tract infections
mAbs	monoclonal antibodies
MCP	monocyte chemotactic protein
MMP	matrix metalloproteinase
Mo	months
(m)RNA	(messenger) ribonucleic acid
NK	natural killer
OLFM4	olfactomedin-4
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PeV	parechovirus

PIV	parainfluenza virus
PMA	phorbol myristate acetate
qPCR	quantitative real-time PCR
ROC	receiver operating characteristic
RPMI	roswell park memorial institute (medium)
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase polymerase chain reaction
RV	rhinovirus
SARS	severe acute respiratory syndrome
SE	standard error
SELDI-TOF-MS	surface-enhanced laser desorption/ionization-time of flight-mass spectrometry
SNP	single nucleotide polymorphisms
TIMP	tissue inhibitors of metalloproteinases
TLR	toll like receptor
TNF	tumor necrosis factor